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CLINICAL CHEMISTRY

Journal of the American Association of Clinical Chemists

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FALL ISSUE, 1957

REACTIONS AND REAGENTS

- *wonderland . . .*
- *inside the heart . . .*
- *forerunner of 17-ketosteroids . . . ?*

Unmasking Plasma Proteins

The White Rabbit *(Oryctolagus cuniculus)* has led many an adventurer on a wond'rous tour. Alice, for one, followed him to Wonderland. Henstell and Feinstein[1] recently followed him in the form of one of our reagents—Simplastin—to some striking findings on clotting deficiencies. Abnormal plasma proteins found in diseases such as cirrhosis, multiple myeloma and metastatic carcinoma, interfere with coagulation by precipitating with proteins (prothrombin and an accelerator factor) necessary in the clotting mechanism. A marked decrease in prothrombin time was noted. Simplastin was the reagent of choice for prothrombin time determinations. When you use Simplastin as your thromboplastin preparation, you need never say, "I'm late, I'm late." Prothrombin times can be done quickly and simply. Your reports are always *on time.*

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1. Henstell, H. H., and Feinstein, M.: Am. J. Med. *22*:381 (March) 1957.

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Our Contribution

The 1956 Nobel Prize in Medicine and Physiology was awarded jointly to Doctors André Cournand,[2] Werner Forssmann and Dickinson Richards for their work in cardiac catheterization. In his acceptance lecture, Dr. Richards[3] stated it took ten years to assemble a comprehensive group of methods for the analysis of cardiopulmonary function, which could be applied not only to the normal but also to patients acutely ill. Among the techniques used was Gregerson's blood volume method using Evans Blue dye. Although not stated in the lecture, Warner-Chilcott supplied Evans Blue Warner-Chilcott in quantitative amounts in sealed ampuls requiring no precalibration of the syringe. Many of the techniques and materials adopted by Cournand, Forssmann, Richards and others are being further developed by circulatory system investigators. Chapman's recent publication on the use of large doses of Evans Blue, introduced into the heart for diagnosing intracardiac and aortic pulmonary shunts,[4] adds to the reports by earlier workers in this field. Evans Blue dye curves, oximetrically recorded, will so reliably identify the site and type of shunts that routine use of Evans Blue is urged for all cardiac catheterizations.

2. Cournand, A. F.: Am. Heart J. *54*:172 (Aug.) 1957.
3. Richards, D. W.: Am. Heart J. *54*:161 (Aug.) 1957.
4. Chapman, C. B., *et al.*: Am. Heart J. *53*:519 (April) 1957.

for blood volume determination

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Tracking Down Steroids

The possibility that certain hormones may undergo conjugation in the liver before they are utilized in the body was investigated in a study of esterification of steroids by surviving liver slices.[5] Ketodase was the beta-glucuronidase used for the hydrolysis. (Ketodase is known for accurate, consistent steroid recoveries time after time.) Since hormones are eliminated as the water soluble conjugates of glucuronic or sulfuric acid, an attempt was made to evaluate the extent steroids are conjugated as glucuronides. Steroids and glucuronic acid were suspended in a media and incubated. Following incubation, the unchanged steroid was extracted. The conjugated steroid was determined either by a specific method for the glucosiduronic acid or by measuring the steroid and the glucuronic acid released from it by hydrolysis with beta-glucuronidase. The study clearly indicates that surviving liver slices are capable of synthesizing glucuronides from a variety of steroids.

5. Sie, Hsien-Gieh, and Fishman, W. H.: J. Biol. Chem. *225*:453 (March) 1957.

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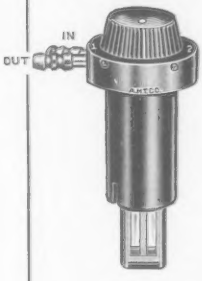

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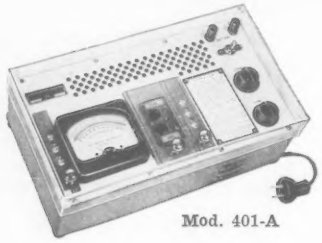

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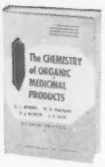

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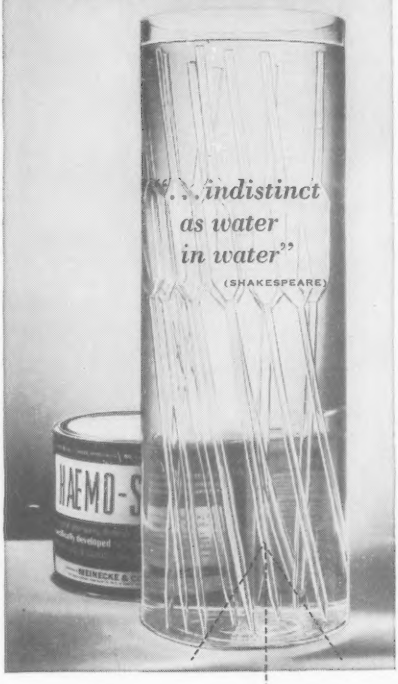

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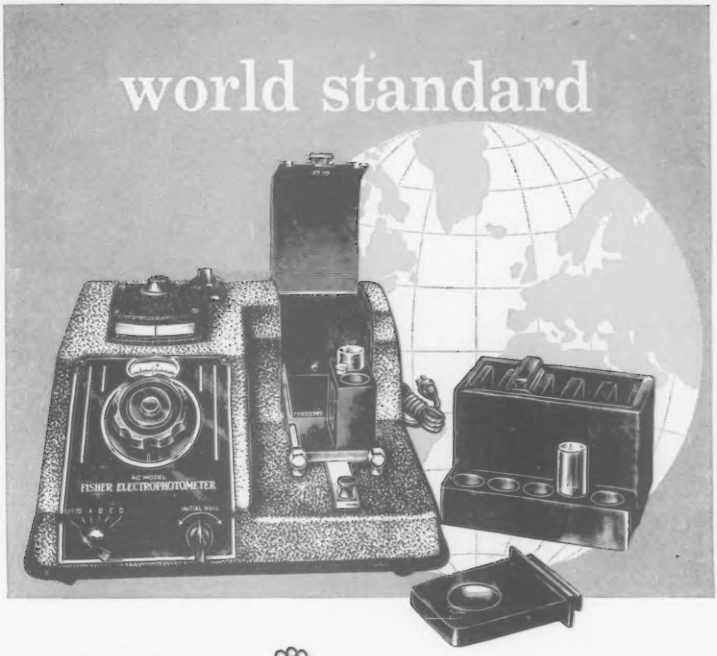

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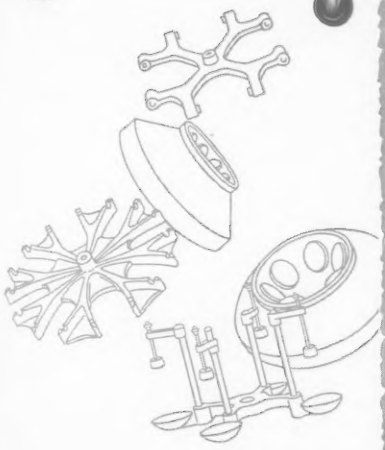

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"U" Protein and Migration Ratios in Paper Electrophoresis

Joel R. Stern, Roland F. Mais, and Joseph D. Boggs

THE PROTEINS OF NORMAL HUMAN SERUM have been resolved both by moving-boundary and filter-paper electrophoresis chiefly into five components: albumin, α_1, α_2, β, and γ globulins. In various disease states, by both technics, other protein components have been described. A component migrating between α_2 globulin and beta globulin has been observed by Block *et al.* (1) in primary systemic amyloidosis and was designated α_2' by these workers. Workers in Japan (2) have noted a similar component in tuberculosis; their protein was named alpha-3 globulin. The term "α_3 globulin," however, was also applied to a fraction which was found in cases of fulminating viral hepatitis and which migrated between the α_1 and α_2 globulins (3). Franklin *et al.* (4) described γ-1 globulin, migrating between beta globulin and the gamma globulin in cases of chronic liver disease. These authors discuss the similarity between their γ_1 globulin and the "T" and "X" components of other investigators (5–8).

This paper is to describe a protein component, migrating between α_2 and β globulin (Fig. 1), which was originally observed in children with liver disease, occasionally observed in sera of normal infants and children, but especially in sera of children with chronic glomerulonephritis and nephrotic syndrome. Alpha$_3$ globulin would be the desirable name for this fraction (2), but α_3 globulin has been

From the Department of Pathology, Children's Memorial Hospital, Chicago, Ill.

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applied to a more rapidly migrating fraction (3); "U" protein will, therefore, be employed to designate this component.

PROCEDURE

Filter-paper strip electrophoresis was carried out in Spinco apparatus using a Durrum-type cell, Whatman 3-mm. paper, 5 to 8 ma. current with terminal voltage of 75 to 78 volts for 30 cm. strips. Unless otherwise specified, veronal buffer *p*H 8.6 ionic strength 0.075 was used. Proteins were stained with bromphenol blue; lipoproteins identified with Sudan black B added to serum before application to paper as suggested by McDonald *et al.* (9) as well as staining of the completed electropherogram (10). The periodic acid–reduced fuchsin technic (11) was employed to identify insoluble carbohydrate-protein complexes. The bromphenol blue–stained strips were scanned with the Spinco "Analytrol," which draws curves relating optical density to distance of migration and automatically integrates the area under the curves. Errors inherent in this system of measurement are within the sphere of accuracy of values obtained.

Control sera were obtained from healthy, 6-week-old infants,[1] from children in the outpatient clinic with minor colds and digestive disturbances and from children with no known disease. Sera considered to be abnormal were from hospitalized children with liver disease, nephrosis, and nephritis.

MIGRATION RATIOS

It became apparent early in the study that the proteins of sera of certain patients, particularly those with the nephrotic syndrome, resolved very poorly. In some instances, "U" protein was clearly visible between the alpha-2 and beta globulins; in other instances these were merely undifferentiated proteins comprising the areas where alpha-2, "U" protein, and beta globulin should be found (Fig. 2). A device similar to the mobility of free electrophoresis, that of migration ratios, was employed to accomplish resolution of these components. [Kunkel (12) has summarized the difficulties in calculating absolute mobilities in supporting media such as filter paper.] Migration ratios are defined as fractions in which the distance from the peak of albumin to the peak of gamma globulin is the denominator and the distance from the albumin peak to the peak of each fraction in ques-

[1]Dr. Iwan Rosenstern and the staff of The Cradle Nursery, Evanston, Ill., generously cooperated in this phase of the study.

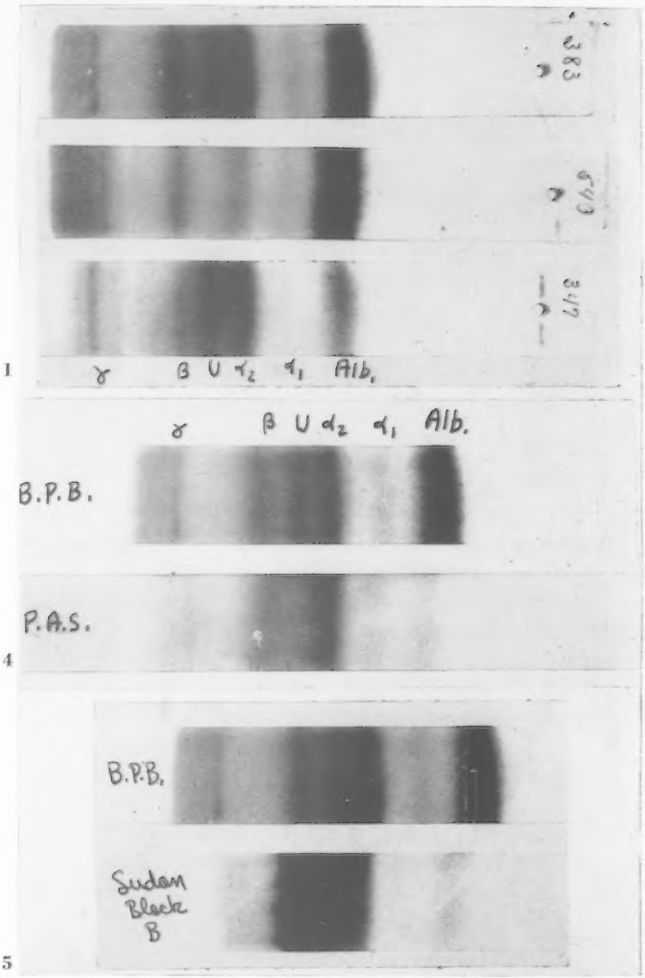

Fig. 1. Stained filter-paper strip electropherograms. Normal serum (*center*) does not contain "U" protein. Sera from children with kidney disease (*top*, chronic glomerulonephritis, and *bottom*, nephrotic syndrome) contain "U" protein, visible between the α_2 and β globulins. **Fig. 4.** Bromphenol blue (BPB) stain and PAS reaction on duplicate electrophoresis strips from the same serum. **Fig. 5.** Bromphenol blue (BPB) and lipoprotein stain on patterns from the same serum.

Table 1. Migration Ratios[a] for Protein Fractions

	Condition							
	Nephritis and nephrosis		*Liver disease*		*"Normal" children*		*Healthy infants*	
	Mean	*No.*[b]	*Mean*	*No.*	*Mean*	*No.*	*Mean*	*No.*
α_1 globulin	0.21	48	0.21	85	0.21	44	0.21	36
α_2 globulin	0.41	48	0.42	85	0.43	44	0.42	36
"U" protein	0.54	23	0.53	32	0.54	14	0.53	3
β globulin	0.68	48	0.66	85	0.67	44	0.68	36

[a] Distance (mm.) from albumin peak to peak of fraction in question divided by distance from albumin peak to γ globulin peak.

[b] 22 patients studied; 48 serum samples analyzed.

Fig. 2. Electropherogram with poor resolution between α_2 and β globulins.

tion is the numerator.[2] Table 1 shows migration ratios in various conditions; the ratios are essentially the same in all. Calculations based upon migration velocities of serum proteins relative to albumin as one (13) yield ratios which agree well with the data of Table 1.

In applying migration ratios to the scan of a paper electrophoresis strip, the distance from the albumin peak to the gamma globulin peak is measured. The migration ratio for each protein fraction is multiplied by this distance, and the product is the distance from the albumin peak to the peak of the protein in question. Figure 3*A* illustrates this process; Fig. 3*B* illustrates the applications of this technic to the pattern shown in Fig. 2. Migration ratios were employed routinely in locating globulin peaks and proved to be a useful device.

CHARACTERIZATION OF "U" PROTEIN

Partial characterization of "U" protein was attempted by staining replicate strips of the same sera for proteins (bromphenol blue: BPB), for protein-carbohydrate complexes (periodic acid leuko-

[2] It is also possible to use the distance from the albumin to the origin, i.e., the point of application of the serum, as the denominator.

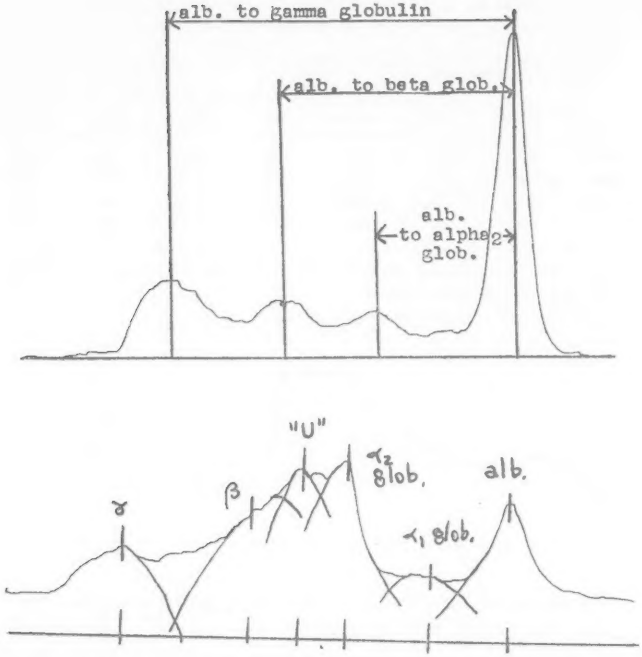

Fig. 3*A*, calculation of migration ratios by locating peaks in normal electrophoresis pattern. ***B*,** Peaks of α_2 globulin, "U" protein, and β globulin located by applying migration ratios to the pattern shown in Fig. 2.

fuchsin: PAS) and for lipoproteins (Sudan black B: SBB). In some cases, "U" protein (BPB) was associated with a clear zone (PAS) between the α_2 and beta globulin, whereas in other instances the PAS pattern closely resembled the BPB pattern. By this technic, therefore, "U" protein was shown to contain varying amounts of protein-carbohydrate complex, differing in this respect from the α_1, α_2, and β globulins, in which protein-carbohydrate complex was invariably present.

When lipoprotein was studied, "U" protein (BPB) typically migrated slightly ahead of the intensely staining β_2 lipoprotein (SBB). Figure 5 (p. 601) shows patterns obtained from sera of patients with the nephrotic syndrome (very high serum lipoprotein and "U" protein) and of patients with liver disease (relatively low lipoproteins

and "U" protein). In both conditions, "U" protein was associated with a lipoprotein band of intermediate intensity. These results indicate that "U" protein may or may not contain protein-carbohydrate complex and that it may be associated with lipoprotein. The α_2 globulin of Block *et al.* (1) (described as not a mucoprotein but possibly lipoprotein) is similar to "U" protein except, perhaps, with reference to the carbohydrate.

BORATE AND PHOSPHATE BUFFERS

Replicate samples of several sera which contained "U" protein in veronal buffer were subjected to electrophoresis in borate and phosphate buffers. Borate buffer causes an appreciably increased mobility of carbohydrate-containing proteins (14) and phosphate buffer gives increased resolution of certain components. Both the borate and phosphate buffers had calculated ionic strengths of 0.075, equal to that of the veronal buffer; the *p*H of the borate was 8.52, and that of the phosphate was 8.59. "U" protein was distinguishable in these buffers in the same relative position as in veronal but was less clear than in veronal.

Table 2 shows that the globulins migrated farther in borate than in barbital buffer; the increase was more pronounced with the alpha globulins. It might be inferred, therefore, that the α globulins may contain more protein-carbohydrate complex than "U" protein and beta globulin. Nothing of interest was noted with the phosphate buffer.

EFFECT OF HEMOLYSIS

A reaction has been described (15, 16) between an α_2 globulin, haptoglobin, and hemoglobin, liberated into serum by hemolysis. The product, hemoglobin-haptoglobin complex, has an electrophoretic mobility less than that of α_2 globulin. It was considered possible that

Table 2. MIGRATION RATIOS IN BORATE AND BARBITAL BUFFERS

	Ratios in borate	*Barbital ratios over borate ratios*
α_{-1} globulin	0.17[a]	1.24
α_{-2} globulin	0.36	1.14
"U" protein	0.50	1.06
β globulin	0.63	1.06

[a] Means of 8 sera.

this complex was responsible for "U" protein; experiments were designed, therefore, to explore that possibility.

Data from children with kidney disease (Table 3) showed that most sera which contained "U" protein were, by visual inspection, hemolyzed. Although there were many exceptions, the possibility arose that hemolysis might have been responsible for "U" protein. The same underlying mechanism responsible for hemolysis in kidney disease, however, may also be responsible for "U" protein.

The hemoglobin-haptoglobin complex was investigated by subjecting serum samples to varying degrees of hemolysis by mechanical agitation while the serum was in contact with the blood clot. These were then centrifuged, the clear serum diluted with 0.1% sodium bicarbonate(0.25 ml. serum plus 2.5 ml. salt solution) and the optical density at 540 mμ read; this gave quantitative knowledge of the degree of hemolysis. The hemolyzed sera were then subjected in duplicate to electrophoresis; "U" protein could then be related to hemolysis (Table 4). There was no indication that hemolysis was a factor in producing "U" protein. Smithies (17) in two-dimensional starch electrophoresis has noted in rare samples of serum from healthy

Table 3. Relation Between "U" Protein and Hemolysis

	Serum with "U" protein		*Serum with no "U" protein*	
	Hemolyzed	*Not hemolyzed*	*Hemolyzed*	*Not hemolyzed*
No. sera	14	11	4	31

Table 4. Effect of Hemolysis on Percentage of "U" Protein

Patient No.		*Per cent of total protein*			
1	Optical Density	0.045	0.065	0.118	0.168
	% "U" Protein	1.7	2.2	2.5	2.5
2		0.060	0.075	0.145	..
		0	0	0	0
3		0.038	0.122	0.163	0.395
		3.0	4.5	3.4	3.9
4		0.075	0.164	..	..
		11.5	15.8	..	..
5		0.041	0.135	0.173	0.282
		0	0	0	0
6		0.035	0.090	0.220	0.410
		0	0		
7		0.035	0.050	0.110	0.600
		4.2	5.3	4.6	4.2

adults a protein with migration characteristics similar to "U" protein. He considers this component to be a physiologic constituent of serum.

In a further attempt to show whether hemoglobin was associated with "U" protein, the benzidine reaction was applied to strips to which various sera, some with and some with no "U" protein, had been applied and subjected to electrophoresis. The blue zone indicative of hemoglobin [and of methemalbumin (18)] was in proximity to the α_2 globulin band.

The weight of the evidence indicates, therefore, that "U" protein represents a true protein component of serum and is not an artifact resulting from hemolysis.

SUMMARY

Paper electrophoresis of serum from children with liver and kidney disease has led to recognition of a protein which migrates between the α_2 and β globulins. This protein has been observed by other workers, but because of confusion in nomenclature, a new designation, "U" protein, has been employed. The use of migration ratios in locating peaks of the α_2 "U" protein, and β globulin when these components resolve poorly has been discussed.

Characterization of "U" protein by employing the periodic acid-reduced fuchsin test for carbohydrate-protein complexes and Sudan black B for lipoprotein showed: (1) "U" protein varies in its content of protein-carbohydrate complex, and (2) "U" protein may be lipoprotein. Evidence based on benzidine tests and intentional hemolysis indicated that "U" protein was not an artifact resulting from accidental hemolysis, but a protein which is found in serum under certain physiological conditions.

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Human Serum Bilirubin

An Immediate Method of Determination and Its Application to the Establishment of Normal Values

J. E. O'Hagan, Teresa Hamilton, E. G. Le Breton, and A. E. Shaw*

In an attempt to detect the carriers of homologous serum hepatitis (HSH), determination of bilirubin in all sera appearing more yellow than normal was instituted at the Red Cross Blood Transfusion Service in Brisbane in 1949. While the estimation did not prove successful as an unequivocal test for HSH carriers, the results brought to light interesting findings both in regard to the methods for estimating bilirubin and to the serum bilirubin levels in normal people.

Most modern methods for serum bilirubin estimation involve diazotization followed by treatment with reagents such as methyl and ethyl alcohol, sodium hydroxide, bile acids, citric acid, urea, caffeine, sodium benzoate, or sodium acetate. In our experience, the widely used methods involving the use of alcohols, such as those of Thannhauser and Anderson (1) and of Malloy and Evelyn (2) and recent modifications thereof, may result in some turbidity in the final solutions, making them particularly unsuitable for use in the determination of low values. Further, they take undue time to perform and involve high dilutions of the azo-pigment.

A modification of the method of Powell (3) was found to be most

From the Laboratories of the Red Cross Blood Transfusion Service, Brisbane, Queensland, Australia.

*Present address: Department of Pathology, Australian National University, Canberra, A.C.T., Australia.

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suitable, and studies to be reported here showed that the method could be made more rapid than had been originally supposed. This modified method was then applied to the determination of the serum bilirubin values of apparently normal blood donors.

REAGENTS AND METHODS

BILIRUBIN

In view of the statement of Lemberg and Legge (4) that commercial bilirubin samples were often of varied purity, a laboratory preparation was made from cattle gallstones by the method of Küster (5). Two batches of crystals were obtained, the first being brownish and glistening, and the second microcrystalline and orange. Later through the courtesy of Dr. John G. Reinhold, an opportunity to examine other preparations of bilirubin presented itself and the E_{mM} at 453 mμ in chloroform and of the azobilirubin in chloroform-alcohol at 532 mμ are given in Table 1. Specimen no. 6, the first batch of crystals from the laboratory preparation, with E_{mM} 532 mμ = 50.0 for the azocompound in alcohol, was used for all the work described in this report. In view of the widely differing values for the various lots, and the findings of Cole and Lathe (6) with regard to the composition of "bilirubin" it would be advisable in future reports to quote the extinction coefficient of the bilirubin used. Bilirubin standards were prepared either by solution in reagent-grade chloroform for comparison of the millimolar extinction coefficients or, for the serum values, by solution in bilirubin-free pooled human serum. The serum was sterile serum prepared for transfusion purposes and over two months old, by which time it was virtually bilirubin free (less than 0.01 mg. per 100 ml.). While such a standard is preferable, it will be shown later that it is not essential, unless very accurate determinations are required for special work.

Table 1. MILLIMOLAR EXTINCTION COEFFICIENTS OF SEVEN BILIRUBIN SPECIMENS

Specimen	*Source*	*Lot*	E_{mM} *453 mμ (free bilirubin)*	E_{mM} *532 mμ (azobilirubin)*
1	Hoffman-La Roche	8508174	56.9	55.6
2	Armour	M470109	58.2	57.6
3	Armour	M480622	59.8	52.4
4	Pfanstiehl	2625	54.3	54.4
5	Eastman Kodak	2101	43.5	42.9
6	Laboratory prep.	1st crystals	48.5	50.0
7	Laboratory prep.	2nd crystals	55.5	52.5

REAGENTS

1. *Solution A.* 1 Gm. of sulfanilic acid AR. is dissolved in a little water containing 15 ml. of concentrated hydrochloric acid AR. and the solution made to 1 liter with distilled water.
2. *Solution B.* 0.5 per cent sodium nitrite AR. solution in distilled water. Keep in refrigerator.
3. *Diazo reagent.* Freshly prepared by adding 0.3 ml. of solution B to 10 ml. solution A.
4. *Diazo blank reagent.* 15 ml. of concentrated hydrochloric acid AR. diluted to 1 liter with distilled water.
5. *Sodium benzoate-urea solution* (*S.B.U.*). 10 Gm. of sodium benzoate C. P. is dissolved in about 60 ml. of distilled water, 10 Gm. of urea (reagent grade) added and dissolved, and the final solution is made to 100 ml. and filtered.

COLORIMETRY AND SPECTROPHOTOMETRY

The E.E.L. Photoelectric Colorimeter, the Unicam D.G. Spectrophotometer, and the Hilger Biochem Photoelectric Colorimeter were used for the routine estimations. The absorption curves, extinction coefficients and the micro estimations were obtained with the Hilger Uvispek Spectrophotometer, (using the glass prism), and the Beckman D.U. Spectrophotometer. The Biochem Colorimeter was found most useful for the kinetic studies.

ROUTINE METHOD OF ESTIMATION

Into two tubes the following volumes were measured:

Tube 1	*Tube 2*
0.50 ml. serum	0.50 ml. serum
0.25 ml. diazo reagent	0.25 ml. diazo blank reagent
4.25 ml. S.B.U. solution	4.25 ml. S.B.U. solution

The volume of S.B.U. added will depend on the particular absorption cuvet used, but should not be less than 4 ml. The methods of standardization of the colorimeters used were in accordance with the principles outlined by O'Hagan (7).

The contents of each tube were mixed and read immediately using a green filter, or in the spectrophotometer at 524 mμ, using the blank to set zero density. Since for most instruments the green filter provided has a transmission peak coinciding with the absorption peak of the azobilirubin complex (Fig. 1) the density/concentration curve is

very nearly linear (Fig. 2) so that it was sufficient to perform one standardization and check at one point from time to time.

For the standardization, 5 mg. of bilirubin were dissolved in 1 ml. of 0.1 N NaOH, and added to about 30 ml. of bilirubin-free pooled human serum. One milliliter of 0.1N HCl was then added and the well-mixed solution diluted to 50 ml. with pooled human serum, using a trace of octyl alcohol on a fine wire to adjust at the finish. From this solution (10 mg. per 100 ml.) 0.5, 1, 1.5, 2, 3, 4, and 5 ml. were taken and diluted to 10 ml. with pooled human serum, the bilirubin diazotized and the optical densities read without delay, the results being

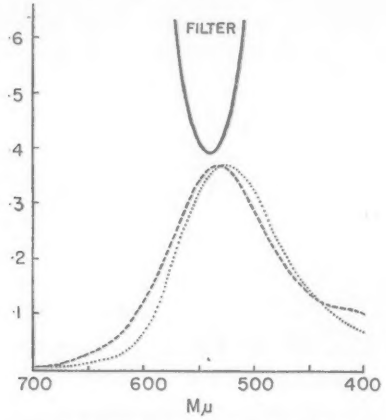

Fig. 1. Absorption curves for azobilirubin complex in buffered serum (. . . .) and in alcohol (– – – –) and for the green filter used for colorimetric estimation.

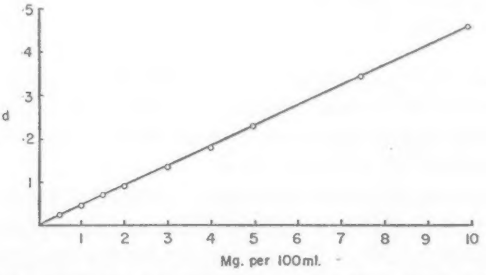

Fig. 2. Calibration curve for colorimeter using green filter, giving a factor of 22 for X, in $X \times d =$ mg. per 100 ml.

shown in Fig. 2. Density values 2 per cent lower were obtained when the estimation was performed by Powell's method using a chloroform solution of bilirubin, 10 mg. per 100 ml., which was diluted 1:12.5 with 95 per cent ethanol. In this case, after standing 30 minutes, the density was read. It is important to check for the presence of reducing substances in the alcohol used. In view of the present difficulties in obtaining bilirubin of definite extinction value it would appear plausible to use this latter standard, which may be more conveniently prepared in some laboratories, where bilirubin-free serum is not readily available.

The method is applicable, of course, to the determination of one-minute bilirubin by substituting water for the SBU mixture.

INFLUENCE OF SUNLIGHT

Although often mentioned in the literature (e.g., Gigon and Noverraz 8, 9) few methods of estimation stress the necessity of keeping solutions of bilirubin out of strong light before determination. It was noted that in some laboratories, sera for bilirubin estimation were often left on the bench for hours before the estimation was performed. To test the destructive effect of sunlight a serum containing 9.0 mg. of bilirubin per 100 ml. was left exposed to direct sunlight for 1 hour (from 2:45 to 3:45 P.M. on a bright spring day). On estimation the value obtained was 4.8 mg. per 100 ml. Another sterile specimen left for 24 hours on a shaded bench dropped from 0.8 mg. to 0.25 mg. for 100 ml., the optical density of the serum at 460 mμ dropping from 0.56 to 0.36 units (Fig. 3). Estimations were therefore performed immediately on drawing the specimen, or after keeping in a refrigerator for up to 2 hours before examination.

DISCUSSION OF METHOD

In view of the fact that full color development occurred within 30 seconds after mixing the reagents it was considered unnecessary to wait for 10 minutes as advised by Powell (Fig. 4). By the time the sera and reagents had been well mixed and the measuring instrument had been adjusted and set to zero optical density with the blank, over 1 minute had elapsed so it was possible to read the unknown without delay. No turbidities were encountered in the many thousands of estimations performed including some on pathologic sera of high globulin content. Since the blank was treated in exactly the same manner as the unknown, (except for the inclusion of the diazo reagent) it was

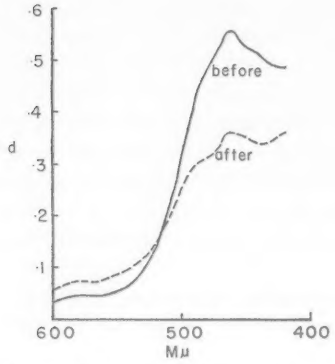

Fig. 3. Destruction of bilirubin by sunlight. Absorption curves for sterile serum before and after exposure to light.

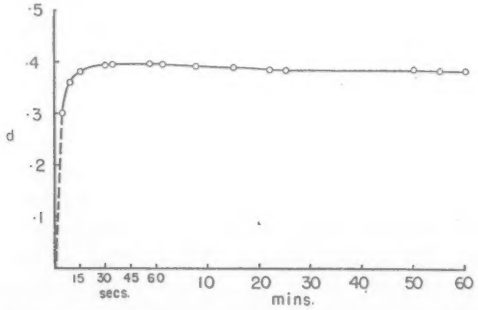

Fig. 4. Rate of development of azobilirubin complex with time on standard serum containing 8.6 mg. bilirubin per 100 ml.

possible to perform estimations in the presence of moderate amounts of hemoglobin, ferrihemalbumin, and especially carotinoids, which latter often account for a major portion of the yellow pigments in serum and which after the addition of the diazo reagent still absorb in the green portion of the spectrum. The method is especially applicable as a micro method. In examining sera of high bilirubin content, where only small volumes are likely to be available, e.g. in infants with icterus neonatorum, the technic works well when 0.1 ml. of serum plus 0.4 ml. saline are substituted for the 0.5 ml. serum and the results multiplied by 5. Very icteric sera may require 3 minutes to develop full color.

RESULTS OF THE USE OF THE METHOD ON BLOOD DONORS' SERA

In the first series of measurements, accurate values were obtained on 200 apparently normal blood donors, judged to be in good health after routine examination by the medical officer and having no previous history of jaundice. The results are shown in Fig. 5, where the mean of values less than 1.0 mg. per 100 ml. was 0.417 with S.D. of 0.178. On splitting this group according to sex, as shown in Fig. 6, the males gave a mean of 0.448 with S.D. of 0.189 and the females a mean of 0.363 with S.D. of 0.178, indicating lower values for females, possibly as the result of a lowered blood hemoglobin content. Furthermore, when the donors who had values above 1.5 mg. per 100 ml. were examined, the proportion of male to female was far in excess of the male preponderance among the donors, which was about two and a half to one (Table 2). During the period the number of males and females admitted to hospital for hepatitis was about equal. The drop in the number with high values after 1953 is due no doubt to our screening program. We somewhat arbitrarily chose the value of 1.5 mg. as the limit of normality, and the 108 individuals who had values

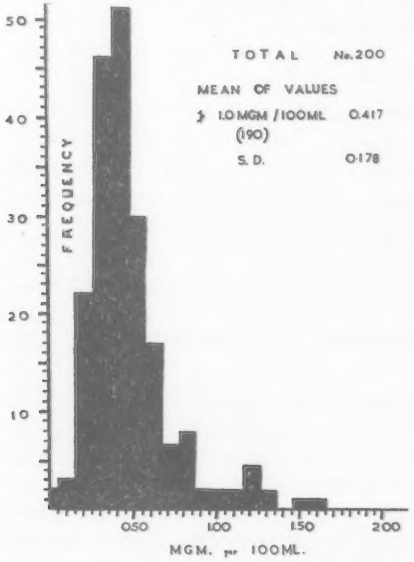

Fig. 5. Histogram showing frequency distribution of serum bilirubin values of 200 apparently normal blood donors. Mean 0.417, with S.D. 0.178.

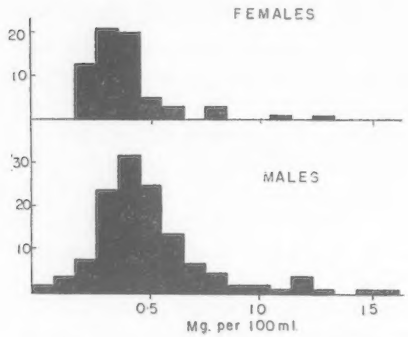

Fig. 6. Histogram showing frequency distribution of bilirubin values from 133 male and 67 female blood donors. Male mean 0.448 with S.D. 0.189. Female mean = 0.363, S.D. = 0.178, determined from values up to and including 1.0 mg. per 100 ml.

Table 2.

Individual donors with serum bilirubin values > 1.5 mg./100 ml.[a]				*Patients with hepatitis admitted to Queensland hospitals*[b]		
Year	*Male*	*Female*	*Total*	*Male*	*Female*	*Total*
1950	10	..	10	214	161	375
1951	11	2	13	180	141	321
1952	32	2	34	398	331	729
1953	33	3	36	346	267	613
1954	5	2	7	306	278	584
1955	3	2	5		Figures not yet	
1956	3	..	3		available	
Total to date	97	11	108	1444	1178	2622

[a]Male/female ratio about two and a half to one. Age group loaded in favor of 18-30 years group.

[b]Figures kindly supplied by Mr. J. E. Solomon, Queensland Government Statistician.

above this limit divide themselves into three approximately equal groups.

In the first group, which comprised 40 individuals, we have placed those whose initial value was greater than 1.5 mg. and from whom we have subsequently been unable to record an observation greater than 1.5 mg. In 29 of these individuals only one observation was made. One developed infectious hepatitis with jaundice and was admitted to hospital four days after visiting the Tranfusion Service. In the remaining ten all subsequent values were below 1.5 mg. over periods ranging from 7 months to 4 years. Of the 40 individuals (36 under 30

years of age) in this category only 4 had a previous history of infectious hepatitis with jaundice, but from 3 to 6 years previously.

The second group comprised those people whose initial observation was below 1.5 mg. and who subsequently recorded a single observation above 1.5 mg. during a total observation period ranging from 1 year to 6 years. There were 38 individuals in this group (3 were females, one of whom developed infectious hepatitis with jaundice four months after the first donation—there was only one male in this group who developed infectious hepatitis with jaundice, having a value of 1.6 mg. per 100 ml. approximately two weeks before diagnosis). In 8 of these individuals the last recorded observation was the high one. The remainder recorded a final figure of below 1.6 mg. and of those, 20 had a value below 1.0 mg., leaving 10 cases whose final values lay between 1.0 mg. and 1.5 mg. (This, we think, is a significantly high proportion and it foreshadows the results to be expected in the third category.) In this second category 27 individuals were under 30 years of age at the commencement of the observation. Two of the individuals in this category had a history of infectious hepatitis with jaundice prior to observation and 2 further individuals developed infectious hepatitis with jaundice during the period of observation. A further individual had a history of jaundice at birth but was Group O, Rh-negative.

In the third group we have placed the thirty individuals who have recorded more than one observation above 1.5 mg. per 100 ml. Table 3 demonstrates the observations on these individuals. It is significant that they are all males. Three had values above 1.5 on seven occasions and the last recorded value on one of these men was 2.1 mg. per 100 ml. Only five had a history of observable jaundice but not within the last 10 years. One donor (P.C.) who was very cooperative and who had been carefully studied had values ranging from 3.1 to 1.0 over the 6-year period. He usually had urinary urobilinogen present in higher concentration than normal, but numerous tests suggested a normally functioning liver.

Elevated serum bilirubin values were not confined to Australian males. The visit of units of the United States Navy in August 1956 enabled the screen to be applied to 44 sailors who volunteered as blood donors. Three were detected with values above 1.5 mg. per 100 ml.—namely, 3.8, 2.8, and 2.1—others had values 1.4, 1.3, and 1.1 mg. per 100 ml. The individual with the 3.8 value had, on electrophoresis of his serum, an elevated γ-globulin but the others were normal or, in one case slightly raised (the 1.1 mg. individual).

Table 3. SERUM BILIRUBIN VALUES OF 30 BLOOD DONORS (ALL MALES) RECORDING MORE THAN ONE VALUE ABOVE 1.5 MG. PER 100 ML

Donor	*Age range of observation*	*History of jaundice*	*When jaundiced*	*No. of values >1.5 mg./100 ml.*	*Last value estimated mg./100 ml.*
1	24-31	+	(1944) at 19 yr.	3	1.0
2	22-29	+	? date	4	2.2
3	20-24		..	3	1.3
4	36-41	+	(1926) at 11 yr.	6	1.6
5	18-21	+	As child	4	1.8
6	24-25	+	(1945) at 17 yr.	3	1.6
7	25-29		..	2	0.7
8	51-57		..	2	0.6
9	21-26		..	3	0.6
10	20-27		..	4	2.0
11	31-37		..	3	0.3
12	21-27		..	3	1.3
13	27-33		..	7	1.0
14	20-26		..	2	1.2
15	18-24		..	2	1.0
16	18-21		..	4	2.0
17	21-23		..	4	1.8
18	18-20		..	4	1.3
19	21-28		..	3	1.0
20	26-28		..	7	1.3
21	23-26		..	3	2.2
22	22-22		..	2	2.5
23	22-28		..	2	0.9
24	21-27		..	2	1.6
25	19-23		..	2	1.5
26	22-28		..	4	1.8
27	26-32		..	7	2.1
28	31-32		..	2	1.6
29	38-42		..	2	1.6
30	18-19		..	2	2.0

SIGNIFICANCE OF A HISTORY OF JAUNDICE

It should not be forgotten that all these investigations have been carried out on individuals whom we have found to be nonanemic and who themselves felt healthy when they first volunteered as blood donors. We were considerably handicapped, of course, by the desire to avoid any suspicion on the part of the blood donors that we were concerned about their health. This did not prevent close questioning concerning a history of jaundice but obviously limited the number and type of investigations which could be carried out. This did not, however, preclude our discovering that practically all these individuals recorded serum protein values that were within the normal range of

6.5 to 8.0 Gm./100 ml. whenever this investigation was carried out. This is contrary to our frequent findings of hypoproteinemia in the serum of our other blood donors. Unfortunately, paper-strip electrophoresis has not been singularly helpful to date. Nor could we find any evidence that cholelithiasis played a significant role in the production of high bilirubin values. A previous history of jaundice was apparently not of much significance (Fig. 7). Accordingly the values on 5540 donors with no previous history of jaundice were plotted and compared with 36 donors who reported having such a history. As can be seen in Fig. 7, no significant correlation was found between the two sets of data that a history of jaundice effected the subsequent bilirubin level, except in the individual who was still recovering from the disease. Again the study of the levels in a fixed age and sex group was undertaken (Fig. 8) using a number of male adults (18-22 years). In this case a significant correlation was possible, but not probable, the P value of 0.02 being obtained. Moreover, we could not find any apparent relationship to homologous serum hepatitis, as none of the

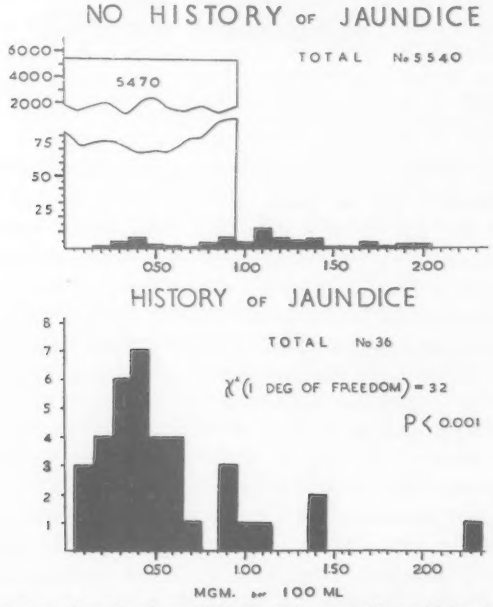

Fig. 7. Histograms of results from 5540 blood donors with no history of jaundice compared with 36 donors with a history of jaundice (highest value—convalescing).

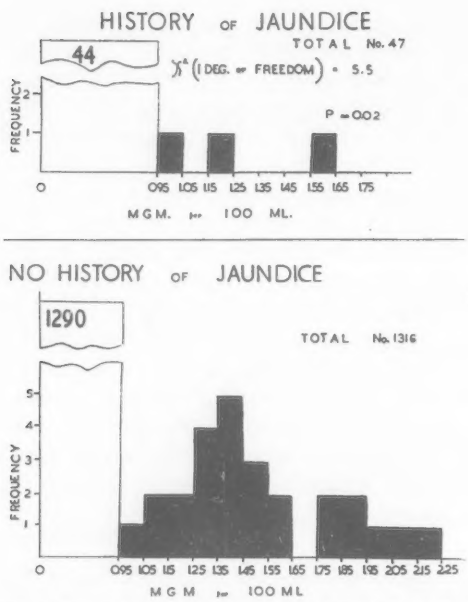

Fig. 8. Histograms of results from 1316 male donors (age 18-22 years) with no history of jaundice compared with 47 donors with history of jaundice.

patients who contracted a suspected homologous serum hepatitis after transfusion could be traced back to donations of blood given from any of the individuals who had recorded a raised bilirubin value, although many of the donations were given immediately prior to the finding of a bilirubin value greater than 1.5 mg. Our practice has been not to issue blood for transfusion if the bilirubin value at the time of donation was greater than 1.5 mg./100 ml. We stress that the investigation is not complete and anticipate that a 20-year follow-up at least will be necessary before the significance of multiple high values can be interpreted.

DISCUSSION

Numerous attempts have been made to determine the normal serum bilirubin value in man. While it was recognized that there had been claims of variations due to age, sex, exercise, fasting, and intake of food (10) some basic level had to be fixed on which to accept or reject a blood donation. The histograms do indicate that levels about 1.5 mg.

per 100 ml. might be looked on with suspicion, and this was accordingly taken as a tentative upper limit of normal. Above this figure donors were not accepted, but were investigated periodically to follow their bilirubin level for indications of developing jaundice. It was then noted however, that these donors with high values were almost exclusively male. With (10) did not consider that sex had an influence on the serum bilirubin levels, though his own values (11) (in mg. per 100 ml.) average 0.71 for men, 0.69 for women; Vahlquist's (12) average 0.82 for men, 0.63 for women; Welin's (13) average 0.50 for men, 0.46 for women; Alwall *et al.* (14) reported an average of 0.56 for men, 0.50 for women. Josephson and Dahlberg (15) give higher figures for men and women, but their results are surprisingly high and would appear to contain a systematic error as suggested by With (10).

There is the possibility that some of the donors with high bilirubin values represent cases of "hereditary non-hemolytic hyperbilirubinemia." Hult (16) considers that cases to which this condition is ascribed are in fact suffering from post hepatic states following manifest or latent viral hepatitis. With (10) regards this as a possibility for the majority of cases but also considers that genuine cases of hereditary nonhemolytic hyperbilirubinemia, though rare, do occur. Of course it would be possible for several members of the same family, particularly if living together during a hepatitis epidemic, to be left with mild liver dysfunction, though it would be strange that the males were affected more than the females. We cannot exclude, of course, the possibility of a familial incidence in at least some of the cases, e.g. those in which a value above 1.5 mg. was recorded several times. Further, we feel there are many who had infectious hepatitis without significant clinical symptoms and without jaundice. Some of these illnesses may have been so mild that the sufferers did not consider it necessary to seek medical attention. It is even more disappointing that only two cases of infectious hepatitis with jaundice had a prodromal bilirubin value greater than 1.5 mg. We will continue to observe individuals with high values, employing clinical tests of liver function and investigating the possibility of erythrocyte abnormalities.

Somewhat similar results to ours were obtained by David (17) and extended by Weiss (18) at the Zürich Tranfusion Centre of the Swiss Red Cross. They used the method of Jendrassik and Gróf (19) which would be expected to give results more comparable to ours than those

obtained with most other technics. Weiss examined 150 donors with values above 1.5 mg. per 100 ml. of whom 34 per cent were women, a value higher than ours, but of the same order of magnitude when compared with the sex ratios in the donor panels. The difference in the male and female values would therefore appear to be, like the raised values generally, not a local phenomenon.

SUMMARY

The technic of Powell (3) for the estimation of serum bilirubin has been modified by reading the optical density of the solutions shortly after mixing the reagents and using a standard based on a more appropriate solution of crystalline bilirubin in pigment-free serum.

Seven specimens of bilirubin were examined spectrophotometrically; the disproportionality of the millimolar extinction coefficients at 453 mμ for the free-bilirubin and at 532 mμ for the azobilirubin complex indicated the presence of an unreactive yellow pigment.

The destructive effect of sunlight on solutions of bilirubin, noted in the literature, was confirmed, hence all estimations were done without delay or exposure to light for any length of time. The results obtained on apparently healthy blood donors, to detect possible carriers of homologous serum hepatitis, revealed no definite correlation between previous history of jaundice and increased serum bilirubin values, but as a precautionary measure individuals with values above 1.5 mg. per 100 ml. were not accepted as donors.

When 200 random values were plotted, the distribution curves gave a mean value higher for males than females. Furthermore, 108 donors (among the 25,000 screened) had bilirubin values greater than 1.5 mg. per 100 ml. The eleven females of the group did not maintain high values, while 30 males recorded high values on more than one occasion, some consistently.

No significant correlation was found between high bilirubin values and a previous history of jaundice, nor were any cases of posttransfusion hepatitis traced to these donors. Somewhat arbitrarily, 1.5 mg. per 100 ml. was chosen as the upper limit of normal.

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Test-Tube Extraction Method for the Micro-Determination of Urinary 17-Ketosteroids

G. R. Kingsley and Gloria Getchell

A SIMPLE, RAPID TEST-TUBE EXTRACTION TECHNIC for the determination of urinary 17-ketosteroids has been developed in our laboratories. A complete urinary 17-ketosteroid determination was carried out in a glass stoppered photometer cuvet by combining this extraction technic with a colorimetric method.

Three colorimetric methods, Zimmermann (1), (2), Pincus (3), and Cohen and Kaluszyner (4) were compared using the test tube extraction technic. A modification of the extraction technic of Drekter *et al.* (5) was used in which choloroform was substituted for ether.

The colorimetric method of Pincus (3) is not as popular as that of Zimmermann or the latter's modification by Callow *et al.* (6) for the determination of 17-ketosteroids. This is unfortunate because of the many difficulties which have been experienced with the colorimetric Zimmermann reaction (7) such as; nonspecificity, sensitivity to the concentration of reagents, slow color development, instability of reagents, nonreproducibility, light and temperature sensitivity, use of color corrections, etc., are avoided by use of the Pincus reaction (3). An objectionable defect of the Pincus reaction is the development of a weak color with dehydroisoandrosterone. However, this ketosteroid, normally constitutes about 10 per cent or less of the urinary 17-ketosteroids. The recently proposed 17-ketosteroid method of Cohen and Kaluszyner (4) in which 3, 5-dinitrobenzoic acid is substituted for *m*-dinitrobenzene gives results, which according to data obtained by these authors represent more accurately the true content

From the Clinical Biochemistry Laboratory, Veterans Administration Center, and the Department of Physiological Chemistry, School of Medicine, Los Angeles 25, Calif.

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of 17-ketosteroids in urine than modifications of the Zimmermann method such as the Nathanson-Wilson (8) and Callow *et al.* (6) methods. 3, 5,-Dinitrobenzoic acid is more stable, gives lower blanks, and develops about the same color densities with both androsterone and dehydroisoandrosterone.

MODIFIED PINCUS METHOD

REAGENTS

Acetic anydride, reagent grade.

Acetic acid-anhydride reagent. Add 1 ml. of acetic anhydride to 9 ml. of glacial acetic acid. Make up fresh shortly before use.

Glacial acetic acid. Reagent grade 99.8 per cent.

Chloroform. Reagent grade.

HCl, Conc. Reagent grade.

10 per cent NaOH.

Antimony trichloride C. P. Use only commercial reagents packed in brown glass-stoppered bottles.

Antimony trichloride-acetic acid-Anhydride reagent. Add 3.8 Gm. of $SbCl_3$ to 1 ml. of acetic acid-anhydride reagent. Weigh quickly in excess of the required amount (for number of tests to be performed) of $SbCl_3$ into a weighing bottle with ground-glass stopper and add acetic acid anhydride reagent in ratio of 1 ml. to each 3.8 Gm. of $SbCl_3$ weighed out. Prepare fresh before use. Warm on steam bath with occasional shaking until clear (about 10 minutes is required).

Androsterone standard (Mann Research Labs. Inc., 136 Liberty Street, New York 6, New York). Dissolve 10 mg. of androsterone in absolute alcohol and dilute to 100 ml. at 5° and store at this temperature.

STANDARDIZATION

Add 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 ml. androsterone standard with microburet to standard cuvets and dilute to 5 ml. with saline. Continue the ketosteroid determination as directed under PROCEDURE for 5 ml. of urine. Plote the $\%T$ of the standards against their equivalent concentration per liter on semilog paper. Make table from the standardization curve to convert $\%T$ to miligrams per liter.

CALCULATION

$$\text{Concentration of total 17-ketosteroids (mg./24 hrs.)} = 38.8\ (\text{2-log per cent } T) \times \frac{\text{24-hr. urine vol.}}{1000}$$

38.8 = K for Junior Coleman #6, B cuvets.

Table 1. Recovery of Androsterone Added to Urine by the Test-Tube Extraction Technic Using the Modified Pincus Colorimetric Method

Tube no.	*Androsterone present (mg.)*	*Mg. added*	*Mg. recovered*	*% Recovery*
1	0.078	0.02	0.096	98
2	0.078	0.03	0.110	102
3	0.057	0.10	0.155	99
4	0.057	0.10	0.155	99
5	0.103	0.02	0.129	105
6	0.103	0.03	0.138	103
7	0.038	0.10	0.124	90
8	0.038	0.15	0.174	93

PROCEDURE

Collect 24-hour urine in a dark bottle with 10 ml. of conc. HCl as a preservative and keep in refrigerator until analyzed[1]. Place 5 ml. of urine and 1.5 ml. of conc. HCl in a 30 ml. ground-glass-stoppered #13, Pyrex colorimeter tube (160 × 19 mm.). Add 5 ml. of saline and 1.5 ml. of conc. HCl in a similar tube for *blank* determination. Heat tubes in a water bath at 80° (temperature of hydrolysate) for 10 minutes. Cool, add 5 ml. of chloroform to each tube, stopper, and shake vigorously for 30 seconds. Draw off aqueous layer with suction tube. Add 2.5 ml. of 10 per cent NaOH and shake vigorously 10 seconds. Siphon off with suction tube alkaline aqueous layer and add 5 ml. of distilled water. Shake vigorously for 10 seconds. Syphon off aqueous layer. Extract twice with 5 ml. of distilled water for 10 seconds or until wash is neutral (*p*H 7). Add one glass bead to each tube and evaporate to dryness in an 80° water bath. Add 0.4 ml. of antimony trichloride-acetic acid-anydride-reagent and mix. Stopper tube, place in boiling water bath and mix thoroughly after standing one minute. Keep tube in boiling water bath for 20 minutes. Cool the tube to room temperature and add 6 ml. of glacial acetic acid. Mix and let stand 40 minutes and read in the photometer against *blank* set at 100 per cent *T* and 615 mμ light transmittance. The color produced is fairly stable between 40 and 60 minutes after start of color development.

EXPERIMENTAL

Good recoveries were obtained with androsterone added both to saline and urine, by the modified Pincus method as shown in Table 1. A recovery of 100 per cent was obtained with mixed standards con-

[1]17-ketosteroids are stable for several days under these conditions.

taining a ratio of 80 per cent androsterone to 20 per cent dehydroisoandrosterone, while 75 per cent of dehydroisoandrosterone was recovered in standards containing equal amounts of these two steroids. The same extraction method as described was used in preparing urine specimens for comparing the Pincus, the Drekter (5), and the Cohen and Kaluszyner (4) colorimetric methods as shown in the data of Fig. 1 to determine the optimum concentration of HCl to use for hydrolysis. Apparently a ratio of 1.5 ml. of conc. HCl for each 5 ml. of urine is optimum for each of the methods. The data of Table 2 represent a comparison of the values of 17-ketosteroids obtained by the different methods on several 24-hour urines. The same test tube hydrolysis and extraction technics as described for the method reported were used in all of the methods compared. The method of Cohen and Kaluszyner (4) was slightly modified for color development as follows:

To hydrolyzed, dried, purified urine extract prepared from 5 ml. of urine, add:

0.2 ml. of methanol and dissolve.

0.2 ml. of 4 per cent 3,5–dinitrobenzoic acid in methanol.

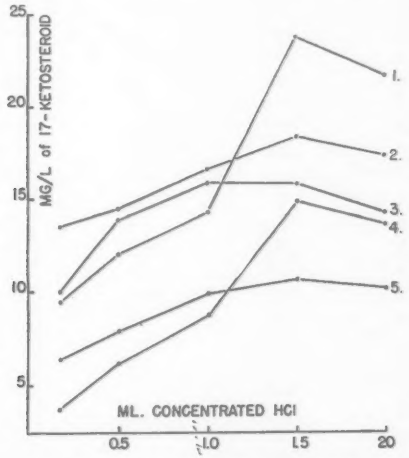

Fig. 1. Determination of milliliters of conc. HCl per 5 ml. of urine for optimum hydrolysis of urinary 17-ketosteroids. *Curve 1,* Drekter method, as androsterone; *Curve 2,* Asher-Kaluszyner method as dehydroisoandrosterone acetate; *Curve 3,* modified Pincus, method as androsterone; *Curve 4,* Drekter method, as dehydroisoandrosterone acetate; *Curve 5,* Asher-Kaluszyner method, as androsterone

Table 2. COMPARISON OF THREE COLORIMETRIC METHODS FOR THE DETERMINATION OF 24-HOUR URINARY KETOSTEROIDS

Urine Spec. No.	*Pincus (Modified)*	*Cohen-Kaluszyner (4)*		*Drekter (5)*	
	Androsterone	*Dehydroiso-androsterone*	*Androsterone*	*Dehydroiso-androsterone*	*Androsterone*
	(mg./24-hr.)	(mg./24-hr.)	(mg./24-hr.)	(mg./24-hr.)	(mg./24-hr.)
1	5.7	7.6	7.0	6.7	3.8
2	13.4	13.3	12.1	17.2	9.4
3	19.4	20.8	19.0	28.6	15.7
4	17.4	19.0	17.3	26.4	14.6
5	25.6	24.0	22.0	31.8	17.6
6	15.6	13.5	12.4	21.8	12.0
7	24.0	22.5	20.8	31.0	17.1
8	10.5	11.0	10.0	35.0	18.8
9	5.9	5.7	5.2	6.7	4.2
10	5.5	3.8	3.5	6.8	3.6
11	8.9	11.8	10.7	26.0	14.3
12	13.1	11.4	10.4	16.1	9.2
13	19.4	17.2	15.6	24.0	13.1
14	13.0	13.4	12.2	22.0	12.2
15	15.4	17.8	16.2	28.0	15.4
16	15.9	18.0	16.4	20.4	11.1
17	14.8	14.8	13.5	18.7	10.3
18	13.4	18.9	15.8	25.0	13.8
19	11.0	8.7	7.9	13.5	7.5
20	8.9	11.0	10.0	16.9	9.4
21	10.3	7.3	6.6	10.5	6.0
22	9.8	8.3	7.5	12.9	7.5
23	8.1	8.9	8.1	8.0	4.4
24	8.1	5.0	4.6	7.0	3.9
25	8.2	9.4	8.5	12.1	6.8
26	11.8	16.2	14.7	25.0	14.0
27	9.8	12.5	11.3	15.1	8.0
28	27.0	22.0	20.0	25.0	14.0
29	10.8	9.5	8.5	11.0	6.2
30	11.8	9.1	8.2	13.6	7.7
31	6.6	6.6	5.9	5.8	3.3
32	12.3	10.6	8.7	16.3	7.9
33	7.9	10.1	8.3	20.7	9.9
34	6.6	6.6	5.7	15.5	7.6
35	12.2	16.3	13.7	28.2	11.8
36	12.0	16.2	13.6	25.2	12.2
37	2.2	2.9	2.3	5.0	2.4
38	17.0	16.7	13.5	29.5	13.9
39	15.4	10.5	8.9	26.6	13.0

0.2 ml. of 5N KOH, mix, let stand for 1 hour at 25° in the dark.

Add 6 ml. of 75 per cent methanol, mix, let stand 4 minutes and read at 550 mμ.

The data in Table 2 indicate that excellent agreement is obtained between the modified Pincus and the Cohen-Kaluszyner (4) methods when standardized with androsterone. Only slightly higher values are obtained by the latter method when dehydroisoandrosterone was used as a standard. Erratic results, as compared to the other methods, were obtained by the Drekter (5) modification of the Zimmermann method, the values ranging from 50 to over 100 per cent higher when measured as dehydroisandrosterone as compared to androsterone standardization. The values obtained by the androsterone standardization of this method also do not agree well with those obtained by the modified Pincus or Cohen-Kaluszyner (4) methods. Most of the values are higher but a few are lower.

The standardization curves for the different methods are shown in Fig. 2.

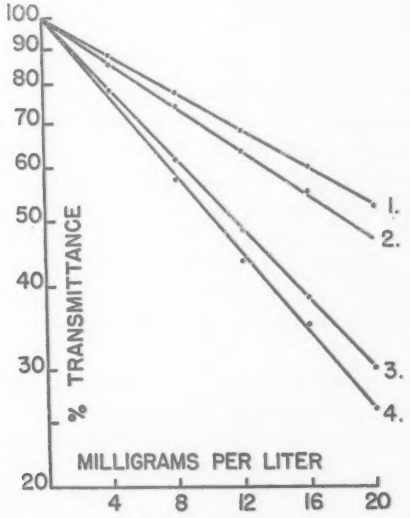

Fig. 2. Standardization curves of the urinary 17-ketosteroid methods compared in this study. *Curve 1,* Drekter (5), Cohen and Kaluszyner (4) dehydroisoandrosterone and dehydroisoandrosterone acetate; *Curve 2,* Cohen and Kaluszyner (4), androsterone; *Curve 3,* Modified Pincus, androsterone; *Curve 4,* Drekter (5), androsterone.

SUMMARY

A rapid, simple, reproducible, test-tube extraction technic requiring less than 90 minutes for the micro-determination of urinary 17-ketosteroids has been presented. Experimental data indicate that the extraction technic may be applied to the Pincus, Zimmermann, or Cohen-Kaluszyner colorimetric methods.

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Practical Evaluation of Acid-Base Balance

Herbert P. Jacobi, Anthony J. Barak, and Meyer Beber

IDEALLY, THE EVALUATION of acid-base balance should be made from the determination of CO_2 content and *p*H on arterial plasma. Obtaining arterial blood is not, however, a routine clinical procedure. Hence, next in order of preference is the performance of these two determinations on plasma of arterialized blood which may be easily obtained by soaking the arm of the subject in a water bath at 40° for 10 minutes and then drawing blood from the antecubital vein (1). This procedure, however, is not used in most clinical laboratories. Instead, quite satisfactorily, venous blood is used, and plasma CO_2 content and *p*H are determined. Frequently, the determination of CO_2 content is performed alone and yields valuable information.

Unfortunately, many hospital laboratories still employ the determination of CO_2 combining power. This determination was developed by Van Slyke and Cullen in 1917 (2). In 1920, Stadie and Van Slyke recommended that the determination of CO_2 content should be used in place of the determination of CO_2 combining power (3). This recommendation was confirmed in 1932 in *Quantitative Clinical Chemistry*, by Peters and Van Slyke (4), the authors pointing out that in their laboratories the CO_2 combining power procedure had not been used for some years. Nevertheless, this determination remains in widespread use.

There are strong practical and theoretical objections to the determination of CO_2 combining power. Because it involves an equilibration with air containing CO_2 at a partial pressure of 40 mm., it is technically more difficult than the determination of CO_2 content. From the standpoint of the physician, it is a poor measure of the in vivo bicar-

From the Department of Biochemistry, University of Nebraska College of Medicine, Omaha, Neb.

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bonate concentration of plasma since it may be either higher or lower and may differ substantially from it; whereas the CO_2 content will invariably be only slightly higher. The purpose of this report is to show the relationship between in vivo plasma bicarbonate concentration and CO_2 combining power in the four types of acid-base balance deviations.

METHODS

The determination of CO_2 combining power was performed by the method of Van Slyke and Cullen (2) and of CO_2 content by the method of Van Slyke and Stadie (5). Blood *p*H was measured at 37° by means of a Beckman glass electrode assembly. Bicarbonate concentration was calculated from *p*H and CO_2 content by means of the Henderson-Hasselbalch equation employing a *p*K of 6.10.

Venous blood was collected anaerobically and plasma obtained from it by the method of Davenport (1). The sample of blood withdrawn was large enough to permit duplicate determinations of each *p*H, CO_2 combining power, and CO_2 content.

Patients with various types of acid-base balance disturbances employed in this study were hospitalized either at University Hospital or at the local county hospital in Omaha, Nebraska. Respiratory alkalosis was produced experimentally in normal subjects who underwent forced breathing for a 15-minute period.

RESULTS AND DISCUSSION

Table 1 presents the results obtained with a group of 10 normal individuals. In each case, it is apparent that the difference between the CO_2 combining power and in vivo bicarbonate concentration is greater than the corresponding difference for CO_2 content. The reason for this becomes apparent when the definition of CO_2 combining power is considered. CO_2 combining power is the concentration of bicarbonate in plasma after it has been equilibrated at 20° with air containing CO_2 at a partial pressure of 40 mm. (4). As a result of the equilibration, the plasma concentration of dissolved CO_2 is raised considerably above the normal concentration because the solubility of CO_2 at 20° is 1.6 times as great as at 38°. The equilibrium points of reactions (*a*) and (*b*) below are, accordingly, shifted to the right, yielding a bicarbonate concentration of plasma significantly greater than that which existed in vivo:

(a) $CO_2 + H_2O \rightleftharpoons H_2CO_3$

(b) $H_2CO_3 + \text{B protein} \rightleftharpoons \text{H protein} + BHCO_3$

Table 1. CO_2 Combining Power and CO_2 Content Compared to Plasma Bicarbonate Concentration in Normal Individuals

		Volumes per cent				
Subject	*pH*	*CO_2 content*	*In vivo [HCO_3^-]*	*CO_2 combining power*	*CO_2 content minus in vivo [HCO_3^-]*	*CO_2 combining power minus in vivo [HCO_3^-]*
1	7.37	55.4	52.6	58.8	+2.8	+6.2
2	7.32	68.8	64.9	69.1	+3.9	+4.2
3	7.36	64.4	61.0	65.3	+3.4	+4.3
4	7.35	59.0	55.9	61.6	+3.1	+5.7
5	7.40	63.5	60.5	65.3	+3.0	+4.8
6	7.46	61.9	59.3	64.3	+2.6	+5.0
7	7.40	57.5	54.8	59.8	+2.7	+5.0
8	7.30	64.8	60.9	65.5	+3.9	+4.6
9	7.31	64.6	60.8	66.4	+3.8	+5.6
10	7.32	62.9	59.3	63.6	+3.6	+4.3

The difference between CO_2 content and in vivo bicarbonate concentration, on the other hand, equals the concentration of dissolved CO_2.

In respiratory alkalosis, the discrepancy between CO_2 combining power and in vivo bicarbonate concentration is even greater than observed for normal plasma. From Table 2, it is apparent that for each subject the difference between CO_2 combining power and bicarbonate concentration is greater after hyperventilation. This result is to be anticipated since in hyperventilation the concentration of dissolved CO_2 in plasma is diminished. The increment in dissolved CO_2 resulting from the equilibration in the determination of CO_2 combining power will, therefore, be greater than when normal plasma is equilibrated, thus allowing for a greater increment of bicarbonate as reactions (*a*) and (*b*) are shifted to the right.

Since in respiratory acidosis the plasma concentration of dissolved CO_2 is above normal, it would be expected that the CO_2 combining power could be less than, equal to, or even slightly greater than the in vivo bicarbonate concentration, depending upon the severity of the respiratory acidosis (Table 3). For a severe case of respiratory acidosis, the equilibration in the CO_2 combining power procedure would result in a decreased plasma concentration of dissolved CO_2. Hence, the equilibrium points of reactions (*a*) and (*b*) would be shifted to the left, resulting in a new bicarbonate concentration which is less than the in vivo bicarbonate concentration. The CO_2 combin-

Table 2. CO_2 Combining Power and CO_2 Content Compared to Plasma Bicarbonate Concentration in Respitory Alkalosis

Subject		*Volumes per cent*				
	pH	*CO_2 content*	*In vivo [HCO_3^-]*	*CO_2 combining power*	*CO_2 content minus in vivo [HCO_3^-]*	*CO_2 combining power minus in vivo [HCO_3^-]*
1 Before hyperventilation	7.30	64.8	60.9	65.5	+3.9	+4.6
1 After 15 min. hyperventilation	7.67	46.6	45.3	54.1	+1.3	+8.8
2 Before hyperventilation	7.31	64.6	60.8	66.4	+3.8	+5.6
2 After 15 min. hyperventilation	7.62	50.2	48.8	57.9	+1.4	+9.1
3 Before hyperventilation	7.32	62.9	59.3	63.6	+3.6	+4.3
3 After 15 min. hyperventilation	7.50	56.0	53.8	64.5	+2.2	+10.7
4 Before hyperventilation	7.34	66.8	63.2	65.5	+3.6	+2.3
4 After 15 min. hyperventilation	7.54	53.3	51.4	59.5	+1.9	+8.1
5 Before hyperventilation	7.35	65.6	62.2	67.3	+3.4	+5.1
5 After 15 min. hyperventilation	7.60	57.0	55.2	63.6	+1.8	+8.4

ing power, accordingly, in this situation would be less than the in vivo bicarbonate concentration.

If, on the other hand, the respiratory acidosis were mild, such that the concentration of dissolved CO_2 were less than that produced after equilibration, the CO_2 combining power would be slightly greater than the in vivo bicarbonate concentration. If the plasma concentration of dissolved CO_2 in respiratory acidosis should happen to equal that obtained by equilibration, the CO_2 combining power and in vivo bicarbonate concentration would be equal.

In metabolic alkalosis, since the plasma concentration of dissolved

CO_2 is increased by the compensatory depression of respiration, the relationship of CO_2 combining power to in vivo bicarbonate concentration is the same as in respiratory acidosis (Table 4).

In metabolic acidosis, since the plasma concentration of dissolved CO_2 is decreased by the compensatory increase in rate and/or depth of respiration, the relationship of CO_2 combining power to in vivo bicarbonate concentration should be the same as in respiratory alkalosis. From Table 5, it is apparent that CO_2 combining power in metabolic acidosis frequently is greater than the in vivo bicarbonate concentration as would be anticipated from the theoretical considerations reviewed above for respiratory alkalosis. However, occasionally, we observed a negative difference between CO_2 combining power and in vivo bicarbonate concentration. Since these negative differences do not appear to stem from a coexisting respiratory acidosis, further work will be necessary to adequately explain them.

It is apparent from this study that the CO_2 combining power bears

Table 3. CO_2 Combining Power and CO_2 Content Compared to Plasma Bicarbonate Concentration in Respiratory Acidosis

		Volumes per cent				
Subject	*pH*	*CO_2 content*	*In vivo [HCO_3^-]*	*CO_2 combining power*	*CO_2 content minus in vivo [HCO_3^-]*	*CO_2 combining power minus in vivo [HCO_3^-]*
1	7.34	88.2	83.4	76.8	+4.8	— 6.6
2	7.37	95.6	90.7	84.3	+4.9	— 6.4
3	7.25	72.5	67.7	62.6	+4.8	— 5.1
4	7.32	76.3	71.9	60.7	+4.4	—11.2
5	7.22	73.4	68.3	57.9	+5.1	—10.4
6	7.20	72.5	67.2	56.0	+5.3	—11.2
7	7.30	74.0	69.6	49.4	+4.4	—20.2
8	7.50	94.1	90.5	89.0	+3.6	— 1.5

Table 4. CO_2 Combining Power and CO_2 Content Compared to Plasma Bicarbonate Concentration in Metabolic Alkalosis

		Volumes per cent				
Subject	*pH*	*CO_2 content*	*In vivo [HCO_3^-]*	*CO_2 combining power*	*CO_2 content minus in vivo [HCO_3^-]*	*CO_2 combining power minus in vivo [HCO_3^-]*
1	7.43	72.4	69.1	60.7	+3.3	— 8.4
2	7.45	70.2	67.3	57.6	+2.9	— 9.7
3	7.52	121.3	116.9	90.5	+4.4	—26.4
4	7.49	122.6	117.8	99.1	+4.8	—18.7

Table 5. CO_2 Combining Power and CO_2 Content Compared to Plasma Bicarbonate Concentration in Metabolic Acidosis

		Volumes per cent				
Subject	*pH*	*CO_2 content*	*In vivo [HCO_3^-]*	*CO_2 combining power*	*CO_2 content minus in vivo [HCO_3^-]*	*CO_2 combining power minus in vivo [HCO_3^-]*
1	7.30	42.6	40.0	43.8	+2.6	+ 3.8
2	7.31	39.8	37.5	42.8	+2.3	+ 5.3
3	7.27	43.6	40.9	39.0	+2.7	− 1.9
4	7.25	50.6	47.2	46.6	+3.4	− 0.6
5	7.22	49.6	46.1	40.9	+3.5	− 5.2
6	7.24	40.3	37.6	35.3	+2.7	− 2.3
7	7.26	38.5	36.0	31.9	+2.5	− 4.1
8	6.90	5.5	4.7	19.2	+0.8	+14.5
9	7.30	44.4	41.8	46.6	+2.6	+ 4.8

a variable relationship to the in vivo bicarbonate concentration, being greater than, less than, or equal to the in vivo bicarbonate concentration depending upon the type and magnitude of acid-base balance deviation. The discrepancy may be so great as to mislead the physician regarding the severity of the acid-base distortion. For example, in cases 3 and 4 of metabolic alkalosis (Table 4), where the CO_2 combining powers were 90.5 and 99.1 volumes per cent corresponding to in vivo bicarbonate concentrations of 116.9 and 117.8 volumes per cent, respectively, the physician would be given a false sense of security regarding the magnitude of the metabolic alkalosis from a consideration of the CO_2 combining power.

CO_2 content, however, bears an essentially constant relationship to the in vivo bicarbonate concentration. It will invariably be slightly higher, the magnitude of the small difference depending on the type and severity of acid-base balance deviation. On the basis of this parallelism, the determination of CO_2 content is to be preferred over the determination of CO_2 combining power in the assessment of an acid-base disturbance.

There are other cogent reasons for this preference. If the *p*H of plasma is known in addition to CO_2 content, it is possible by calculation or with a nomogram to ascertain bicarbonate concentration and the partial pressure of CO_2. This useful information cannot be derived from a knowledge of *p*H and CO_2 combining power. The CO_2 combining power, moreover, since it may deviate considerably from

the in vivo bicarbonate concentration, cannot be used in diagrams showing the relationship of bicarbonate concentration to other plasma ions such as sodium or chloride. These considerations, together with the fact of the greater technical simplicity of the determination of CO_2 content, should recommend the abandonment of the determination of CO_2 combining power.

SUMMARY

The CO_2 combining power bears a variable relationship to the in vivo plasma bicarbonate concentration, depending upon the type and severity of acid-base distortion. In respiratory alkalosis and metabolic acidosis the CO_2 combining power will usually be greater than the in vivo plasma bicarbonate concentration; whereas, in respiratory acidosis and metabolic alkalosis the CO_2 combining power will usually be less. CO_2 content, on the other hand, will always parallel the in vivo plasma bicarbonate concentration quite closely, being only slightly greater. These facts, together with other considerations which are discussed, recommend the abandonment of the determination of CO_2 combining power.

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Determination of Sulfobromophthalein in Serum

David Seligson, Jean Marino, and Elizabeth Dodson

SINCE THE INTRODUCTION of sulfobromophthalein (BSP) as a test for liver function, (1) it has gained wide acceptance and is considered (2) the most reliable single test for evaluating liver function. BSP extraction by the liver has also been used as a measure of hepatic blood flow.

Measurement of BSP in water solution is relatively simple in that the compound is a dye, purple in alkali, and colorless in acid. The difference in intensity of color when *p*H is changed from alkaline to acid is proportional to concentration. In serum, the presence of turbidity, bilirubin, or hemoglobin interferes with the analysis because these factors change absorbance with sharp changes of *p*H and are falsely read as BSP. Gaebler (3) made corrections for these factors by reading optical densities at 580 and 620 mμ and applying a correction. Reinhold (4) made readings at 420, 580, and 660 mμ to correct for hemolysis, jaundice, and turbidity. The method to be described reduces the number of colorimetric readings to a single wavelength. It eliminates interference from the above factors by minimizing the *p*H change to the minimum required for the color measurement of BSP. Furthermore, the effect of protein-binding of BSP is eliminated by adding a large excess of anions to the reaction mixture.

APPARATUS

1. *Photometer.* For analytical work, measurements were made in a Bausch and Lomb monochromatic colorimeter using a 575 mμ inter-

From the Department of Biochemistry and Internal Medicine of the Graduate School of Medicine of the University of Pennsylvania, Philadelphia, Pa.

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ference filter and an 18 mm. test tube cuvet. The latter requires a minimum of 4.0 ml. of colored solution. Adaptations to smaller or larger cuvets or other photometers can be made readily. Spectral-absorbance curves were obtained on the Beckman DU Spectrophotometer.

REAGENTS

1. *BSP standard, 10.0 mg. per 100 ml.* This solution is equivalent to the 100 per cent retention standard for the 5 mg. per kilo test dose of BSP (5). Pure BSP can be obtained from Hynson, Westcott and Dunning in Baltimore as "Bromsulphalein." Their test solution which is readily available, contains 50.0 mg. per ml. It is diluted 1 to 500 to make the 10.0 mg. per 100 ml. standard. (A personal communication from Dr. John Brewer indicates that this test solution varies from 98 to 102 per cent of the stated value and is, therefore suitable for standarization of the BSP method).

2. *Alkaline buffer* p*H 10.6-10.7.* Dissolve 12.2 Gm. of $Na_2HPO_4 \cdot 7H_2O$, 1.77 Gm. $Na_3PO_4 \cdot 12H_2O$ and 3.20 Gm. sodium *p*-toluenesulfonate and make to 500 ml. with water. Adjust to *p*H 10.6 to 10.7 with 1 N NaOH or 1 N HCl.

3. *Acid reagent, 2M NaH PO₄.* 69 Gm. of $NaH_2PO_4 \cdot 1H_2O$ dissolved in distilled water and diluted to 250 ml.

PROCEDURE

Place 0.50 ml. of serum into a test tube cuvet and add 3.5 ml. of alkaline buffer. Mix gently and read in the photometer at or near 580 mμ using water as a reference. Add 0.10 ml. of acid reagent. Mix gently and read again. Proceed to the next sample. Determinations can be made on 0.1 ml. of serum using proportionally less of the reagents and reading in micro cuvets. In this laboratory 0.1 ml. serum, 0.7 ml. buffer and 0.02 ml. acid reagent was read in a 1 cm. micro cuvet at 580 mμ in a Beckman Model B Spectrophotometer.

Calculation is based on the 10.0 mg. per 100 ml. or 100 per cent retention standard put through the method as described.

$$\text{mg. BSP per 100 ml. serum} = \frac{\text{mg. per 100 ml. of standard}}{\text{optical density of standard}} \times \text{optical density of serum.}$$

$$\text{Per cent retention (for 5 mg. per kilo dose)} = \frac{\text{Per cent retention standard} \times \text{optical density of serum}}{\text{optical density of standard}}$$

The calculation of retention for the 2 mg. per kilo dose can be made by similar adjustments of standards. If the rate of disappearance of dye and per cent retention is desired, the formula of Nadeau (6) may be used since retention is proportional to concentration.

$$K = \frac{\log R_1 - \log R_2}{t_2 - t_1}$$

where K is the velocity constant

R_1 is the per cent retention at time 1 (t_1) in minutes.
R_2 is the per cent retention at time 2 (t_2) in minutes.

STUDY OF THE METHOD

In order to determine the dissociation curve of BSP, 10 ml. of a 0.5 mg. per cent solution in 0.01M NaH_2PO_4 were placed in a 1 in. x 2 in. shell vial and then placed into a photometer so that absorbance at 580 mμ could be measured. Small increments of 1N NaOH were added to the reaction mixture from a Scholander microburet. Absorbance was measured with each change in *p*H. The data so obtained is plotted in Fig. 1. The $_pK$ for the dye is 8.8 and 98 per cent of color is obtained between *p*H 7.4 and 10.4. For purposes of analysis these *p*H limits were used in the determination of BSP in order to attain maximum color with minimum *p*H change. Table 1 shows the *p*H range achieved by this method as compared to the methods of Rosenthal (7), Gaebler (3), and Reinhold (4).

A series of optical density measurements was made on serums which contained no BSP, but which were clear, hemolyzed, jaundiced, or turbid in order to ascertain the effect of *p*H changes on optical

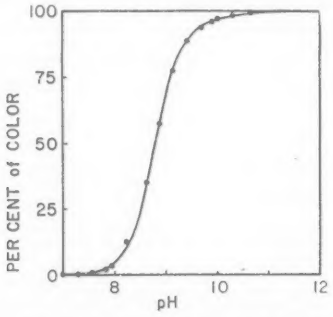

Fig. 1. Dissociation curve of BSP.

Table 1. *p*H Changes Obtained During BSP Measurements in Four Methods

Method		*No.*	*Mean*	*σ*
Reinhold	Serum[a]	35	8.31	0.34
	Serum + NaOH		11.82	0.18
	Δ *p*H		3.52	
Gaebler	Serum[a]	36	8.31	0.34
	Serum + NaOH		11.87	0.12
	Δ *p*H		3.57	
Rosenthal	Serum + NaOH	4	10.86	0.07
	Serum + HCl		6.52	0.10
	Δ *p*H		4.34	0.12
Proposed	Serum + alkaline buffer	36	10.48	0.16
	Neutral mixture		7.12	0.14
	Δ *p*H		3.36	0.16

[a]Data were obtained from 30 serums open to air 2-4 hours after clotting. These high values were not caused by hydrolysis of urea. *p*H's were checked against buffer standards of *p*H 7.00 and 9.36 at 29°C.

density. The data shown in Table 2 indicate that greater optical density changes (calculated as per cent retention) occur in the Rosenthal (7), Gaebler (3), and Reinhold (4) methods where *p*H changes are uncontrolled (see Table 1). These changes introduce errors in the analysis which Gaebler and Reinhold (3, 4) correct by factors. The proposed method shows less interference when *p*H changes are controlled.

It was observed that the hue of the BSP at *p*H 10.3 was purple but became more pink when albumin was present. Figure 2 demonstrates

Table 2. Optical Density Changes Recorded as BSP in Clear, Hemolyzed, Jaundiced, and Turbid Serum Free of BSP in Order to Demonstrate the Effect of Interfering Factors

		Per cent retention					
		Reinhold		*Gaebler*		*Proposed*	
Condition of serum	*No.*	*Mean*	*Range*	*Mean*	*Range*	*Mean*	*Range*
Clear	10	0.87	0.0-2.3	1.08	0.0-2.5	0.7	0.0-2.0
Hemolyzed[a]	8	2.6	1.3-6.7	3.4	1.6-7.0	1.5	0.3-4.0
Jaundiced[b]	8	1.1	(—) 1.6-3.8	1.4	0.3-5.5	0.4	0-2.5
Turbid[c]	2	3.9	(—) 3.6-4.1	2.9	1.6-4.2	0.7	0.2-1.2
Bilirubin Std.[d]	4	1.6	(—) 1.4-(—)1.8	1.3	1.1-1.7	0.0	0.0
Hemoglobin Std.[e]	2	1.6	(—) 0.5-2.7	1.6	0.3-3.0	0.0	0.0

[a]27 to 138 mg. per 100 ml. hemoglobin by measurement. [b]1 to 30 mg. per 100 ml. bilirubin by measurement. [c]Grossly turbid. [d]2 and 5 mg. per 100 ml. bilirubin. [e]13 and 137 per 100 ml. hemoglobin.

the spectral-absorbance curve of BSP at *p*H 10.3 without and with albumin. The maximum absorption occurs at 580 mμ in the absence of serum and 594 mμ in its presence. Furthermore, there was less absorbance in the latter case. When albumin solutions simulating serum samples were put through the analytical procedure, and where toluenesulfonate was omitted from the alkaline buffer, a loss of optical density was observed with increasing amounts of albumin (see Figs. 2 and 3).

In order to avoid this protein effect which could be troublesome since serum proteins vary widely in liver disease, a strong anion, *p*-toluenesulfonate, was added to the buffer. The albumin in serum is approximately 0.6 micromol per ml. or in the reaction mixture of this method 0.07 μmol. per ml. The BSP at 100 per cent retention is 0.12 μmol/ml. in serum or 0.015 μmol. per ml. in the reaction mixture. Albumin in normal serum is 5 times the molar concentration achieved by BSP at 100 per cent retention. *p*-Toluenesulfonate as added in the procedure reaches 29 μmol. per ml. in the final reaction mixture, which is 400 times the molar concentration of the albumin which would be

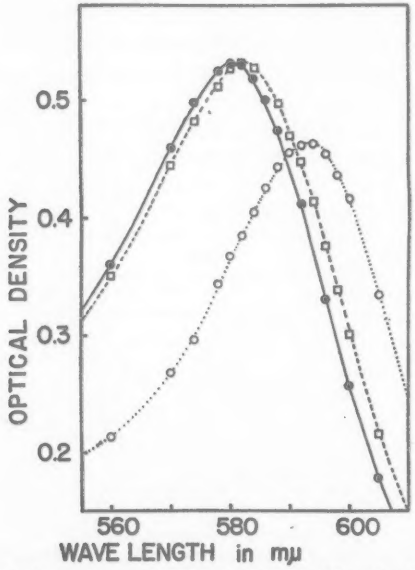

Fig. 2. Spectral-absorbance curve of BSP at pH 10.3 with and without albumin. Black dots = BSP; open squares = BSP + albumin + PTSA; open circles = BSP + albumin.

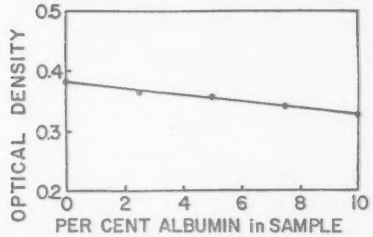

Fig. 3. Effect of albumin on optical density of BSP in serum. The abscissa represents Gm. per cent of an albumin[1] solution containing BSP (25 per cent retention) which was run as a hypothetical serum (0.5 ml. albumin solution plus 3.5 ml. alkaline buffer).

present in the serum sample, and 900 times the BSP. When *p*-toluenesulfonate is present, the serum effect on the spectral absorbance curve almost disappears as shown in Fig. 2. The suppression of optical density also disappears. The sample of serum containing BSP measured with alkaline buffer free of *p*-toluenesulfonate gives 87 per cent of the optical density obtained with equal amounts of BSP in water or in serum as shown in Fig. 3. The standard curves obtained with serum BSP show the same difference (89 per cent) between those with and those without *p*-toluenesulfonate.

Comparison of the proposed method with the method of Reinhold (4)[2] is shown in Table 3. The results are similar except for relative discrepancies in the lower concentrations and where marked hemolysis occurs.

DISCUSSION

The measurement of the simple dye BSP in the presence of albumin, bilirubin, hemoglobin, and turbidity can be difficult. Since measurements are most often made in serum, albumin is always present, more or less. In liver disease the albumin may vary from 0.5 to 5.0 Gm. per 100 ml. of serum. When turbidity is present, usually due to lipoproteins and lipids, some clearing may be achieved at high *p*H such as occurs when NaOH (3, 4, 7) is used for alkalinization. When acidification to low *p*H with HCl (7) occurs, the turbidity may recur and actually be more than that originally present in the serum sample.

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[1] Human serum albumin as provided by the American Red Cross for therapeutic use.

[2] Data provided by Dr. John Reinhold of the Pepper Laboratory of the University of Pennsylvania.

It should be noted that hemoglobin, in serum which slowly becomes more alkaline, could change sufficiently so that hemoglobin corrections[3] made for freshly drawn serum might not apply to serum which aged during transfer between laboratories.

Table 3. COMPARISON OF VALUES OF BSP RETENTION OBTAINED BY REINHOLD AND BY PROPOSED METHOD

	Per cent retention		*Per cent retention*
Case	*Reinhold2*	*Proposed*	*R. — P.*
1	1.0	0.0	0.0
2	4.0	0.8	3.2
3	4.0	1.6	2.4
4	5.0	5.7	—0.7
5	6.0	6.5	—0.5
6	6.0	4.8	1.2
7	6.0	4.8	1.2
8	6.0	5.5	0.5
9	6.0	7.1	—1.1
10	7.0	4.0	3.0
11	7.0	7.0	0
12	8.0	3.5	4.5
13	8.0	5.7	2.3
14	9.0	6.3	2.7
15	10.0	7.2	2.8
16	12.0	10.3	1.7
17	13.0	11.1	1.9
18[a]	15.0	5.5	9.5
19	15.0	12.2	2.8
20	15.0	14.8	0.2
21	16.0	12.3	3.7
22	21.0	17.7	3.3
23[a]	22.0	8.3	13.7
24	27.0	26.2	0.8
25	31.0	28.6	2.4
26	45.0	43.0	2.0
27	48.0	42.6	5.4
28	69.0	70.8	—1.8

[a] Badly hemolyzed. See text.

This problem is accentuated by delaying readings between alkaline and acid reaction mixtures. Since BSP is measured at a wavelength (580 mμ) near where turbidity changes are measured, interference is not easily eliminated. The net effect of turbidity change from alkali to acid would be to give a falsely low BSP measurement. Hemoglobin present in serum resulting from hemolysis is converted in alkali to alkaline hematin. Over a broad *p*H range it causes changes depending upon time of exposure which can lead to errors. Bilirubin changes absorbance slightly between high and low *p*H so that jaundiced serums yield absorbance shifts with change of *p*H.

In the proposed method the *p*H shift is between 10.4 and 7.1. Over

this *p*H range there appears to be little or no absorbance change due to turbidity, jaundice, or hemolysis. Furthermore, in the presence of a large amount of *p*-toluenesulfonate no albumin effect is noted. When measurements are made as described the effects are essentially eliminated. Control of these factors favorably affect accuracy in the BSP measurement and reduce artifacts.

A series of serums were analyzed by the proposed method and by the method of Reinhold (4). The latter is Gaebler's method with a third correction factor for hemoglobin.[3] The proposed method obtained lower values in the low range where normal retentions occur. This may be due to less artifactual interference,[2] although these readings are easily reproduced in this method. Furthermore, as shown above, serums with no BSP, even though hemolyzed, icteric, or turbid, do not give false readings.

Speed is achieved since no corrections for interfering substances need be made and no readings at two or three wavelengths need be made. Specificity appears to be increased by controlling *p*H.

The effects of albumin, as shown in Figs. 2 and 3, are negated by the anion *p*-toluenesulfonate when added in large amounts. It is assumed that albumin binds BSP and this binding is prevented by the presence of the *p*-toluenesulfonate.

SUMMARY

A method for the determination of BSP is presented which eliminates errors due to protein-binding with albumin and artifacts due to bilirubin, hemoglobin, and turbidity. Measurements at two or three wavelengths are thereby reduced to measurement at one wavelength. The method while simplified appears to be accurate, particularly in the low ranges.

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[3] BSP mg. per 100 ml. $= f\ (U_{565} - 1.28\ U_{660}) - 0.15\ (U_{420} - 1.95\ U_{660})$ where U_{420}, U_{660} are absorbances at 420, 565 and 660 mμ, and f is the $\frac{\text{concentration of BSP.}}{\text{absorbance at 565 m}\mu}$.

Determination of Ketone Bodies in Blood and Urine by Means of Vanillin in Alkaline Medium

Victor E. Levine and Michael Taterka

In a previous communication (1) we have reported that vanillin reacts with a large number of organic compounds with a carbonyl group, with aldehydes, and particularly with ketones, diketones, and ketonic acids to produce yellowish, or yellowish-red color complexes. Because of its wide reactivity the vanillin-alkali reagent cannot be used for any specific carbonyl compound unless it is first separated by extraction with solvents, by precipitation, by chromatography, by electrophoresis, or by distillation or aeration especially if the compound has a low boiling point. Acetone has a low boiling point, 56.5°. It can therefore be separated easily by aeration or distillation from other compounds in a biologic fluid or solid tissue. Acetoacetic acid may also be determined, for upon treatment with acid it is readily converted to acetone. The ketogenic acid, β-hydroxybutyric acid, also yields acetoacetic acid on oxidation. We have developed a method for acetone based upon its separation from blood and urine by distillation and subsequent treatment of the distillate with a vanillin-alkali reagent. This method offers many advantages over the very widely used procedure of Behre and Benedict (2) in which salicylaldehyde in alkaline medium is utilized as the reagent.

Salicylaldehyde or 2-hydroxybenzaldehyde is a compound similar in chemical reactivity to vanillin or 3-methoxy-4-hydroxybenzaldehyde. The formation of a reddish-colored complex, the sodium salt of

From the Department of Biological Chemistry and Nutrition, The Creighton University School of Medicine, Omaha, Neb.

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Present address (M. T.): Abbott Laboratories, North Chicago, Ill.

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dihydroxydibenzalacetone, resulting from the interaction of acetone and salicylaldehyde was first reported by Fabinyi (3) in 1901. This reaction was later utilized by Rosenthaler (4), Frommer (5), and Leffmann (6) as a qualitative test for acetone in urine, and by Deshons (7) for acetone in spinal fluid.

The salicylaldehyde-alkali reaction was first suggested by Czonka (8), in 1916, as a quantitative method for acetone. Behre and Benedict (2), in 1926, reported a similar method for ketone bodies. Ravin (9), in 1936, published a criticism of the Behre and Benedict procedure and proposed modifications to overcome the disadvantages of their technic, and in 1940, Behre (10) modified the original Behre and Benedict procedure to overcome the objections raised by Ravin.

Vanillin (3-methoxy-4-hydroxybenzaldehyde) is a compound possessing many chemical properties in common with salicylaldehyde. Both are aromatic aldehydes and both possess a phenol group. Vanillin possesses several important advantages over salicylaldehyde. The former is a white crystalline compound that can be weighed accurately. It is fairly soluble in water, but not too soluble to prevent its purification by crystallization from water. Salicylaldehyde is a liquid, insoluble in water, deteriorating very rapidly and developing a deep brown color on standing even for a short time in a brown bottle. For use as a reagent it requires purification by distillation. Behre and Benedict (2) found great differences in the delicacy of the color given by different samples of salicylaldehyde reacting with acetone. Ravin (9) and Nadeau (11) also noted marked variations in the intensity of the color produced by different brands of salicylaldehyde. Furthermore, to be utilized as a qualitative or quantitative reagent for acetone, salicylaldehyde must be dissolved in alcohol. The use of alcohol as a solvent complicates matters, unless the alcohol is also purified to remove carbonyl-containing compounds by means of silver salts (silver acetate) and alkali (sodium hydroxide), or better still by means of 2,4-dinitrophenylhydrazine subsequent to distillation. Such carbonyl-bearing compounds also produce colored complexes with salicylaldehyde as well as with vanillin in alkaline medium.

Vanillin is more stable, although, according to Englis and Manchester (12), a dilute solution of vanillin (10 Gm. per liter in 10 per cent ethyl alcohol) is slowly oxidized to vanillic acid, the oxidation being fairly complete in several weeks. Solutions of vanillin do not darken on standing. Fresh solutions, if need be, can be readily and quickly prepared. Concentrated alkali added to salicylaldehyde pro-

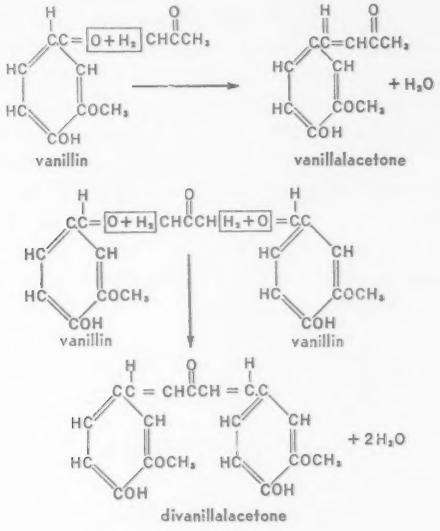
duces a yellow color at room temperature. The yellow characteristic color produced by acetone and salicylaldehyde with concentrated alkali is necessarily an addition to the color produced by salicylaldehyde and alkali alone. Vanillin solutions in the cold do not take on color upon the addition of alkali. Many investigators have utilized vanillin as a reagent in a medium made acid with more or less concentrated solutions of sulfuric acid. Vanillin yields a characteristic yellow color with sulfuric acid without the aid of heat. Nikitin (13) made a comparison of the reactivity of acetone with vanillin and with salicylaldehyde in equal concentrations of alkali. He found vanillin to be 5.27 times more reactive than salicylaldehyde. Of the 103 compounds—aliphatic, alkyl-aryl, and aryl ketones, cyclic ketones, ketone carboxylic acids, aliphatic and aromatic aldehydes, aldols, ketols, acetols, and some α-hydroxyacids—we have investigated with the vanillin-alkali reagent, acetone proved to be the most sensitive and the most intensely reactive (1).

While neither the salicylaldehyde-alkali reaction nor the vanillin-alkali reaction is specific for acetone, we can take advantage of its relatively low boiling point (56.5°) and volatility to separate it from other reacting compounds. Removal of the acetone from biologic fluids is necessary because of the interfering color development with alkali alone or with the vanillin-alkali reagent. A urine sample was acidified with sulfuric acid and boiled until the sample was free of acetone and acetoacetic acid. When cooled the heated urine was treated with the vanillin-alkaline reagent. The reaction mixture turned light brown. Addition of alkali alone produced the same result as heating. Furthermore, certain nonvolatile compounds, such as pyruvic acid and other α-ketonic acids, also react with vanillin in alkaline medium (1). Pyruvic acid also reacts with the salicylaldehyde-alkali reagent (14).

REACTION OF VANILLIN WITH ACETONE

Vanillin condenses with acetone to form vanillalacetone or divanillalacetone. Glaser and Tramer (15) prepared the sodium salt of vanillalacetone by treating vanillin and acetone in alkaline medium. The sodium salt crystallized as yellow needles. Vanillalacetone itself formed beautiful broad yellow-white needles melting at 129°. From a medium acidified with hydrochloric acid Glaser and Tramer prepared divanillalacetone as reddish-yellow or orange crystals, which

lost water of crystallization at 100°. The anhydrous compound formed green prismatic crystals melting at 155-160°.



QUALITATIVE METHOD

Acetone (plus diacetic acid) can be qualitatively detected in urine or blood by means of a procedure similar to the one adopted by Behre (10). Instead of her reagent, salicylaldehyde, we have substituted vanillin. In our procedure 5 ml. of urine or protein-free blood are placed in a test tube and 1 drop of 1:1 sulfuric acid added. A small thin square of cotton is treated in the center with one drop of 1% vanillin solution and two drops of 10N sodium hydroxide solution. The cotton square is inserted in the test tube with the spot reagent facing downward. The tube is placed upright in boiling water for 5 minutes. In the presence of the ketone bodies, acetone, or diacetic acid, the reagent in the cotton plug assumes a yellow or yellowish-red or reddish color depending upon the quantity of ketone bodies present.

Because of the high volatility of acetone, it was thought possible that the Conway microdiffusion technic (16) could be adopted for the detection of acetone. Such a diffusion method for acetone has been described by Werch (17). Acetone solutions as well as urines con-

taining definite amounts of acetone were introduced into the outer chamber of the Conway unit. Into the center of the Conway unit were placed 1 ml. of vanillin solution and 2 ml. of 40% sodium hydroxide. The lid of the unit was sealed with petroleum jelly, and the unit incubated at 37° for two hours. Using acetone standards of widely varying concentrations the mixture in the inner well containing the vanillin-alkali reagent developed a pale orange color. After 24 hours with comparatively larger concentrations of acetone, long reddish needles crystallized out. The Conway diffusion technic with the vanillin-alkali reagent has the possibility of being worked out as a quantitative method for acetone in biologic fluids. Winnick (18) has developed a diffusion method for acetone in blood and urine based on the Conway diffusion procedure. The acetone is trapped in sodium bisulfite, liberated from the aldehyde-sulfite addition product and titrated with 0.005N iodine. Thin and Robertson (19) recently developed a diffusion method for the ketone bodies, utilizing salicylaldehyde and 4N potassium hydroxide as the reagents.

QUANTITATIVE METHOD

Nikitin and Vershinsky (20), in 1937, reported a quantitative method for acetone by means of vanillin in a highly concentrated alkaline solution. Their method, published in Russian, was not developed specifically to meet biologic requirements, and is not a spectrophotometric method. In order to develop a quantitative biochemical procedure of a photometric nature we must determine: (1) the sensitivity of the reaction, (2) the absorption spectrum in order to set the photometric apparatus at the proper wave length as indicated by the peak in the absorption spectrum, (3) the ability of the reaction system to follow the Beer-Lambert law, and (4) the extent of recovery of a definite quantity of the compound added to biologic material.

We have found the vanillin-alkali reaction for acetone to be sensitive to 2 μg. Figure 1 gives the absorption spectrum with a peak at 420 mμ. The calibration curve in Fig. 2 is indicative of the fact that the reaction follows the Beer-Lambert law. Table 1 shows the percentage of recoveries of added quantities of acetone from blood and from urine.

REAGENTS

All the reagents should be made up with doubly distilled water free from the iron and copper compounds, which act as catalysts of oxida-

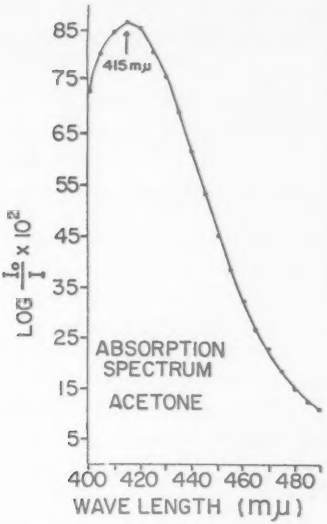


Fig. 1. Absorption spectrum of acetone.

Table 1. Analysis of Blood and Urine

	Acetone and diacetic acid[a] *(mg./100 ml.)*	*Acetone added (mg./100 ml.)*	*Acetone found (mg.)*	*Acetone recovered (mg.)*	*% Recovered*
Blood					
Sample 1	2.60	2.50	4.97	2.37	94.8
		25.00	27.92	25.32	101.3
Sample 2	2.50	14.00	16.30	13.80	98.6
Sample 3	2.00	5.00	6.85	4.85	97.0
		7.00	8.90	6.90	98.7
		14.00	16.20	14.20	101.4
Urine					
Sample 1	0.55	0.50	1.06		100.2
		1.50	2.12	1.55	104.6
		1.20	1.79	1.24	103.3
Sample 2	0.70	0.50	1.22	0.51	102.0
		1.00	1.68	0.98	98.0
		3.00		3.08	102.7
		7.00	7.60	6.90	98.6
Sample 3	0.80	5.00	5.65	4.85	97.0

[a]Measured as actone.

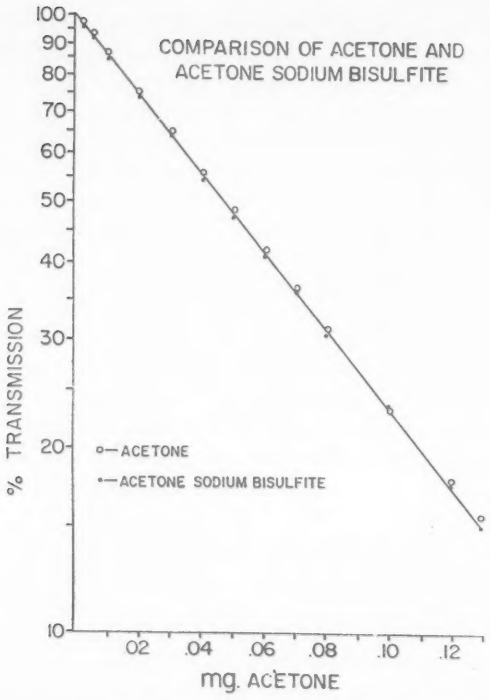


Fig. 2. Graph indicating compliance with the Beer-Lambert law.

tion. The distilled water should be freshly boiled and cooled to room temperature. The precaution of boiling is to remove oxygen which may be dissolved in the water in order to minimize the oxidation of vanillin to vanillic acid. These precautions improve the keeping qualities of the vanillin reagent.

1. *Vanillin reagent.* Vanillin (Eastman-Kodak) is recrystallized from hot distilled water. With the dry crystals a solution of 0.72% vanillin is prepared. This solution corresponds to a 0.047 molar solution of vanillin. We have found that a 1% solution of vanillin tends partially to crystallize out on standing.

The vanillin should be kept in a brown bottle with a glass stopper. We have tested the reagent after a month and found that it had not deteriorated during that length of time.

2. *Sodium hydroxide* (10N). This solution is prepared by dissolving 400 Gm. of C.P. sodium hydroxide pellets in cooled freshly distilled water and diluted to 1 liter.

3. *Sulfuric acid.* 1:1 sulfuric acid, reagent grade.

4. *Sodium bisulfite.* A freshly prepared 2.0% solution made from analytic grade reagent.

5. *Acetone,* purified by distillation, if necessary.

To standardize acetone, 5 ml. acetone solution are placed in a 250 ml. glass-stoppered flask. At first 25 ml. 1N sodium hydroxide solution are added and then 50 ml. 0.1N iodine solution. This solution should be measured with great accuracy. The iodine solution is added a little at a time and the container shaken after each addition. The flask is stoppered and allowed to stand for 10 to 15 minutes. Normal sulfuric acid is added to the extent of 26 to 30 ml. In order to liberate iodine, the acid must be in excess of the alkali present in the mixture. Acetone reacts with the iodine to form iodoform. Excess iodine is titrated with 0.1N sodium thiosulfate, using starch solution as indicator. The quantity of 0.1N iodine originally added minus the quantity of unused 0.1N iodine equals the quantity of 0.1N iodine that reacted with acetone. One milliliter of 0.1N iodine is equivalent to 0.9675 mg. acetone.

A standard acetone stock solution is made up so as to contain 1 mg. of acetone per milliliter. This standard stock solution should be kept in the refrigerator in a glass-stoppered bottle. It does not deteriorate for a month. Freshly prepared dilute standards are made by diluting 0.1 ml., 0.5 ml., 1.0 ml., and 5 ml. of the standard solution to 100 ml. with distilled water. These dilute standards contain respectively 1 μg., 5 μg., 10 μg., and 50 μg. of acetone per ml. Acetone bisulfite equivalents respectively are 2.774 μg., 13.8735 μg., 27.747 μg., and 138.735 μg. per ml.

PROCEDURE

For the estimation of acetone and diacetic acid in normal persons, 10 ml. of urine and at least 10 ml. of protein-free blood filtrate are taken for analysis. This filtrate is obtained by the Folin-Wu procedure (1 volume of blood, 7 volumes of water, and 1 volume of 10 per cent sodium tungstate, and 1 volume of 2/3N sulfuric acid). For pathologic blood or urine containing relatively large quantities of acetone and diacetic acid, less volume may be employed.

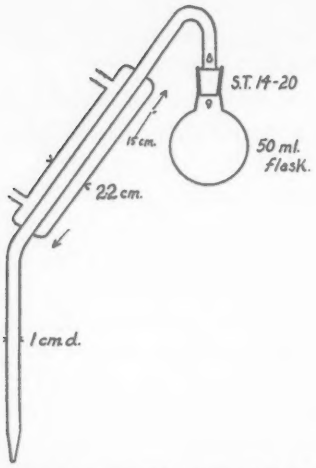


Fig. 3. The Stotz distillation apparatus (Macalaster-Bicknell Co., Cambridge, Mass.)

The urine or protein-free blood filtrate, after the addition of several glass beads and 3 drops of 1:1 sulfuric acid, is distilled in order to separate the volatile acetone. The all-glass apparatus described by Stotz (21) has been found suitable. The neck of the distilling flask in the area nearer the flask is plugged with a small quantity of glass wool as an additional precaution to prevent bumping, especially when urines are used. The end of the condenser is arranged to dip below the surface of 1 ml. of 2 per cent sodium bisulfite contained in a 15 ml. graduate cylinder or a 15 ml. graduate tube immersed in ice water. The sodium bisulfite solution has been introduced to avoid loss of volatile acetone by the formation of a nonvolatile addition compound. Klein (22) has shown that the bisulfite reaction can be used for the quantitative determination of acetone and diacetic acid in blood filtrates free of sugar. The dissociation of the carbonyl-bisulfite compound can be repressed by using a large excess of sodium bisulfite and lowering the temperature to 5°. Winnick (18) has devised a method for acetone based on the bisulfite-addition compound.

Distillation is carried out with a micro burner at a moderate heat for a period of 2 minutes to yield a total of about 4.5 ml. in the collecting container. The condenser is lifted out of the distillate and about 0.5 ml. is distilled off to rinse the condenser. The surface of the condenser that was dipped into the distillate should also be rinsed and the

washing added to the receiver. The total volume in the receiving tube should be 5 ml.

The contents of the receiving tube are thoroughly mixed, and a 1 ml. aliquot (equivalent to 0.20 ml. of blood or 2.0 ml. urine), is transferred to a graduated test tube. One milliliter of vanillin solution and 2 ml. of 40% sodium hydroxide are added. The contents of the tube are thoroughly mixed by gentle shaking, and the tube placed in a water-bath at 60° for exactly 15 minutes.

The mixture is finally diluted with distilled water to 6 ml. and read in a spectrophotometer at 415 mμ.

SUMMARY

Vanillin in an alkaline reaction mixture is a very sensitive reagent for acetone and diacetic acid. A qualitative and a quantitative method have been developed for the acetone bodies in blood and in urine by means of a vanillin-alkali reagent. Acetone and diacetic acid are distilled off and caught in a sodium bisulfite solution, which is allowed to react with vanillin in alkaline medium to form a color complex, which is measured spectrophotometrically.

Vanillin has many advantages over the salicylaldehyde method of Behre. These have been enumerated in the text.

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Determination of Uric Acid

A Note

E. R. Hausman, George T. Lewis, and J. S. McAnally

We have developed in this laboratory a modification of the Kern and Stransky (1) method for the determination of uric acid which is similar to that of Archibald (2).

Two ml. of serum or plasma is added to 14 ml. of water, and 2 ml. of 10 per cent sodium tungstate (molybdenum free) and 2 ml. of 2/3N H_2SO_4 are added successively. The precipitate is removed by filtration. Five milliliters of the filtrate are pipetted into a test tube (5 ml. of water serves as a blank and 5 ml. of uric acid solution (0.004 mg./ml.) is used for a standard). To each tube is added 2.6 ml. of a sodium silicate-glycerine solution (6 parts of 10 per cent sodium silicate and 1 part of glycerine), the solutions are mixed, and 0.4 ml. of Benedict's uric acid reagent is added. After mixing, the solution is allowed to stand for 15 minutes for color development. The color is stable at the maximum intensity for 10 minutes. Photometer readings are made using a 620 mμ filter.

When the procedure outlined is followed rigorously there has been no interference with turbidity, and the results obtained have compared favorably with accepted uric acid determinations employing sodium cyanide. No special reagents other than the sodium silicate-glycerine solution are required, and all reagents are stable for long periods without refrigeration.

From the Department of Biochemistry, University of Miami, School of Medicine, Coral Gables, Fla.

Received for publication June 4, 1957.

This method has proved to be direct, simple, and trouble-free, and the results obtained have in every case been satisfactory.

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BOOK REVIEWS

Methods of Biochemical Analysis. D. GLICK (Ed.). New York, Interscience Publishers, Inc., 1956, Vol. 3, 437 pp., $9.50; Vol. 4, 1957, 362 pp., $8.50

The third and fourth volumes of Glick's *Methods of Biochemical Analysis* maintain the high standard of this annual series. More than half of the contributions originate in this country, the remainder in Great Britain, Scandinavia, and Switzerland. Rather than selecting articles at random for discussion, it should be mentioned that both volumes again contain material of importance for the practicing clinical chemist. The extensive chapter on flame photometry by Margoshes and Vallee is most useful, even though their multichannel flame spectrometer will have to remain at present, a "daydream" for the routine laboratory worker.

The article by Raaflaub on "Application of Metal Buffers and Metal Indicators in Biochemistry" extends the theory of *p*H of Michaelis and Sørensen, in slightly too brief a fashion to *p*M, the negative logarithm of metal ion concentration. The practical analytic application deals exclusively with calcium, and forms a good theoretic basis for currently developed complexometric titration methods of this element.

Among sections in Vol. 3 of practical interest to the clinical chemist, we wish to mention the short chapter on the enzymatic determination of uric acid and other purines, in which Plesner and Kalckar give a micromethod for uric acid by uricase that may be worthwhile testing as a routine procedure.

The preceding essay deals with histamine; besides its biologic assay, a moderately intricate method of the authors is described, using dinitrofluorobenzene, which appears to yield accurate results in the μg. range. Although the authors of this article and those of the last article of Vol. 4 explain their abbreviations at the first occasion where they occur, this reviewer does not cherish symbols like "CS" for "cotton acid succinate," "CB" for "cotton benzoate," and their like; such ad hoc abridgements are introduced without general consensus and often without regard for misleading duplications; moreover, they conflict with the established chemical nomenclature, the beautiful simplicity of which they tend to undermine. That they may be avoided altogether, without disadvantage to printer or reader, is witnessed by the style of most other articles in the volume. It is our belief that such abbreviations should remain confined to laboratory protocols, a criticism not aimed specifically at the present authors or the editors [cf. R. V. Ormes, *Science* **125,** 794 (1957)].

The articles on determination of carotene and of vitamin A cover a wide territory; thus, the author of the first one does not get around to mentioning a method for that determination of carotene in blood, and the second, while giving a method for both vitamin A and carotene in blood from the literature, will not satisfy the clinical chemist with an account that omits, for example, the normal range of values. It is regrettable that the authors of these two articles did not get together on the numbering of the carbon chain; it would almost appear that opposite numbering systems are officially approved for these classes.

A description of determination of 17,21-dihydroxy-20-ketosteroids is most welcome for the clinical laboratory. The article on the *p*H-stat from Carlsberg Laboratory abounds with valuable suggestions. The last four articles in Vol. 4 deal with various enzymes, amongst which the acid *p*H serum phosphatase should be of great interest to clinical chemists. While the King-Armstrong method and its various modifications are the most popular procedure for the evaluation of this enzyme, a description of the Bodansky method using glycerophosphate would have been in order, especially because of its value for the differentiation between prostatic and the recently discovered lipidosis phosphatase.

The volumes of this series adhere in general to the precepts for authors and editors such as have been laid down by Hume-Rothery [*Research* **10,** 168 (1957] and R. O. Kapp (*The Presentation of Technical Information,* London, Constable and Co., 1948) and also by J. R. Baker [*Nature* **176,** 851 (1955)] and Sheridan Baker [*Bull. Am. Ass. Univ. Prof.* **42,** 464 (1956)]. The procedures described may be performed without further consultation of the original literature. The same editorial care should apply to the style and to such stilted verbiage as "With regard to . . ., the most important consideration is that of maintaining accuracy . . . ." and "The decision of ascribing normality to a certain number of units of enzyme activity is based on a number of considerations . . . ." should not be permitted to mar the style of these excellent volumes. Aside from these minor criticisms, they are prepared with the meticulousness which we are used to expect from the editor and the publishers, and they can be heartily recommended.

Mount Sinai Hospital, New York, N. Y. HARRY SOBOTKA

ABSTRACTS

Editor: ELLENMAE VIERGIVER. *Contributors:* JOSEPH S. ANNINO, GLADYS J. DOWNEY, CLYDE A. DUBBS, ALEX KAPLAN, MARGARET M. KASER, MIRIAM REINER, HERBERT THOMPSON

Urinary Port-Silber chromogen versus blue tetrazolium chromogen as a quantitative index of adrenocortical function. L. J. Marks, J. H. Leftin, and M. P. Leonard (*Veterans Hospital, Boston, Mass.*).

The daily urinary excretion of Porter-Silber chromogen and blue tetrazolium chromogen have been determined by a method employing β-glucuronidase hydrolysis and chromatography in normal adult males, male patients with endocrine diseases, and male patients following the administration of corticotropin. The correlation between the methods was poor in all cases. Urinary Porter-Silber chromogen has been shown to be a more specific quantitative measurement of adrenocortical function in man than urinary blue tetrazolium chromogen.—*J. Clin. Endocrinol. and Metabolism* **17,** 407 (1957). (M. R.)

Clinical applications of the fractionation of serum proteins by paper electrophoresis. F. W. Sunderman, Jr., and F. W. Sunderman (*Jefferson Medical College, Philadelphia, Pa.*).

Alterations in the electrophoretic pattern of serum proteins in various diseases are reported for approximately 1000 patients.—*Am. J. Clin. Pathol.* **27,** 125 (1957). (H. E. T.)

The excretion of bilirubin as a diglucuronide giving the direct van den Bergh reaction. B. H. Billing, P. G. Cole, and G. H. Lathe (*University of London, London, England*).

The direct-reacting bile pigment is a mixture of two components, pigment I and pigment II. Pigment I, which occurs with pigment II in serum and urine, appears to be the monoglucuronide of bilirubin. Pigment II, the major one in human bile, is the diglucuronide ester of bilirubin. The difference in the type of van den Bergh reaction and in the physiological behavior of bilirubin and pigments I and II can be accounted for by the fact that bilirubin is lipid-soluble, whereas the bilirubin glucuronides are water-soluble.—*Biochem. J.* **65,** 774 (1957). (A. K.)

A method for the determination of aldosterone, cortisol and corticosterone in biological extracts, particularly applied to human urine. P. J. Ayres, O. Garrod, S. A. Simpson, and J. F. Tait (*Middlesex Hospital, London, England*).

A method is described for the analysis of aldosterone, cortisol, and corticos-

terone after continuous extraction of human urine at *p*H 1 with chloroform. Extensive purification of the steroids by column chromatography is followed by paper chromatography. The appropriate spots on the chromatogram are measured quantitatively by fluorimetry after development of fluorescence with a sodium hydroxide solution.—*Biochem. J.* **65,** 639 (1957). (A. K.)

Pharmacological and biochemical study of a carcinoid tumor. N. J. Giarman, V. S. Green, J. P. Green, and M. K. Paasonen (*Yale University School of Medicine, New Haven, Conn.*).

The appendix removed from a woman at surgery consisted of normal tissue except for a carcinoid tumor at its tip. 5-hydroxytryptophane decarboxylase activity was determined in both tissues. The amount of 5-hydroxytryptophane formed per Gm. of tissue per hour was 840 μg. for the tumor and 23 μg. for the normal appendix. By bioassay technics the carcinoid tissue was found to contain nearly 1000 times as much serotonin as the appendix.—*Proc. Soc. Exp. Biol. Med.* **94,** 761 (1957). (M. M. K.)

The determination of microgram quantities of protein in biological fluids: The estimation of plasma and serum protein in spinal fluid. G. R. Kingsley and G. Getchell (*School of Medicine, University of California at Los Angeles, Calif.*).

The influence of time, temperature, *p*H, and medium upon the reaction between protein and tetrabromophthalein ethyl ester (TBPEE) were studied. The optimal conditions were correlated and methods described for determination of protein in serum and cerebrospinal fluid. According to the authors, cerebrospinal fluid protein binds 10 to 14 times as much TBPEE as serum protein. A formula is given for calculating the amount of serum protein in abnormal spinal fluid.—*J. Biol. Chem.* **225,** 545 (1957). (J. S. A.)

Isolation and identification of a new ketone body in normal human urine. M. U. Tsao and E. L. Pfeiffer (*University of Michigan Medical School, Ann Arbor, Mich.*).

Approximately 50 mg. of the 2,4-dinitrophenylhydrazone of an unknown neutral ketone were isolated from 10 liters of urine from 5 healthy adults. It was shown to be identical with the 2,4-dinitrophenylhydrazone of methyl ethyl ketone by the lack of depression of the mixed melting point, by paper chromotography with methanol-saturated heptane, and by the absorption spectra in 0.25 N NaOH in 70 per cent alcohol. The likely source of methyl ethyl ketone is believed to be the catabolism of isoleucine, although a dietary origin is possible.—*Proc. Soc. Exp. Biol. Med.* **94,** 628 (1957). (M. M. K.)

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